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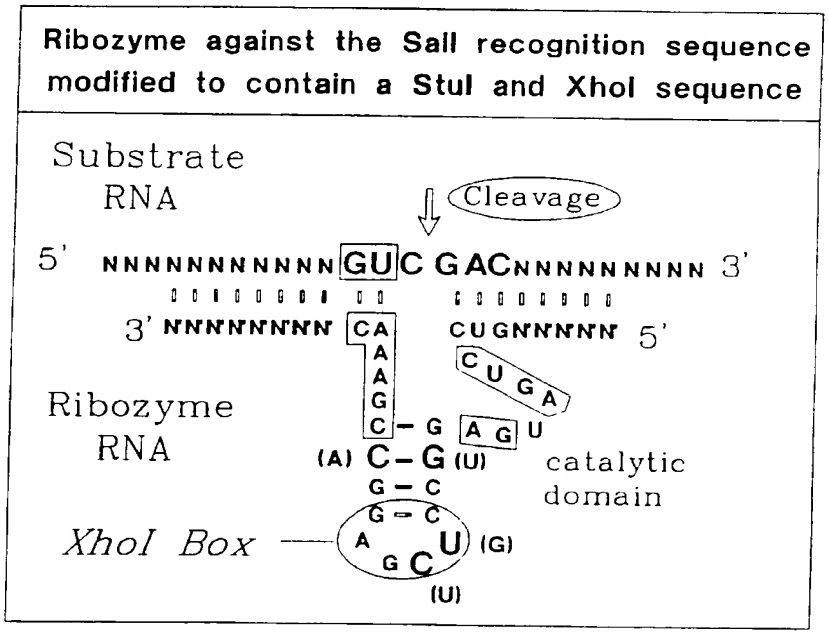

(57) Abstract

Portable ribozyme cassettes are described which can be incorporated into restriction enzyme cleavage sites of any desired DNA sequence. Thus, highly selective ribozymes (antizymes) ." provided. Additionally, methods for the production of these portable ribozyme cassettes, DNA sequences containing them and encoding biologically active ribozymes are described. Also described are compositions containing such ribozymes or DNA sequences encoding them.

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Portable ribozyme cassettes, DNA sequences containing them, ribozymes encoded by these DNA sequences, and compositions containing these ribozymes

The present invention relates to portable ribozyme cassettes (which may also be designated as "insertable ribozyme cassettes") of general applicability which can be inserted into given restriction enzyme cleavage sites and can thus be used for the simplified construction of ribozymes. The invention also relates to DNA sequences containing said portable ribozyme cassettes, recombinant vectors containing such DNA sequences and to host organisms which are transformed by the recombinant vectors of the present invention. Furthermore, the invention relates to the ribozymes encoded by said DNA sequences. The invention also relates to compositions containing the ribozymes or DNA sequences of the present invention. Other embodiments will become apparent from the following description.

Enzymes which are composed of an RNA chain only, are called "RNA enzymes" or "ribozymes". Such catalytic RNAs have been observed in different biological systems.

The first ribozyme discovered is the nuclear rRNA from Tetrahymena thermophila which contains an intervening sequence (IVS) of 413 nucleotides, and is capable of undergoing self-cleaving in the absence of proteins. The IVS catalyzes different transesterification reactions, which result in the excision of the IVS from the precursor RNA and the ligation of the two exons. This IVS RNA enzyme has been described in detail, e.g. Kruger at al., Cell 31 (1982); 147-157, Cech, Cell 34 (1983), 713-713; Zaug et al., Science 224 (1984), 574-578; Cech, Cell 44 (1986), 207-210; Cech and Bass, Annu. Rev. Biochem. 55 (1986), 599-629; Zaug et al., Nature 324 (1986), 429-433; Zaug and Cech, Science, 231 (1986), 470-475; Cech, Science 236 (1987), 1532-1537; Latham et al., Method. Enzymol. 181 (1990), 558-569.

Cech and coworkers have also described the modification of the IVS ribozyme to use it for the cleavage of large RNA molecules in a sequence-specific manner. The modified IVS recognizes small sequence motifs, like CUCU and related sequences like CCCU and cleaves them in a sequence-spe-

cific manner comparable to DNA restriction enzymes.
A precise description of the ribozymes of Cech et al., in particular the usage as a sequence-specific endoribonuclease, can also be found in WO 88/04300.

The ribozymes of the present invention are derived from another class of naturally occurring RNAs which undergo site-specific autolytic cleavage generating two cleavage products having a 5'-hydroxyl group and a 2',3'-cyclic phosphodiester, at their termini, respectively. The majority of these self-cleaving RNAs originates from satellite RNAs of plant viruses. There is also one self-cleaving RNA of this type found in another plant pathogen, the avocado sunblotch viroid (ASBV) and in an RNA transcript of newt (reviewed by Bruening, Method. Enzymol. 180 (1989), 546-558). A self-catalyzed cleavage reaction has been demonstrated in vitro for the avocado sunblotch viroid (ASBV) (Hutchins et al., Nucleic Acids Res. 14 (1986), 3627-3640, the satellite RNAs of tobacco ring spot virus (sTobRV) (Prody et al., Science 231 (1986), 1577-1580; Buzayan et al., Proc.Natl.Acad.Sci. 83, (1986), 8859-8862), of lucerne transient streak virus (sLTSV) (Forster and Symons, Cell 49 (1987), 211-220) and also for RNA transcripts of repeated DNA sequences from newt (Epstein and Gall Cell 48 (1987), 535-543).
All these self-cleaving RNAs can assume a so-called "hammerhead" structure (Forster and Symons, Cell 50, (1987), 9-16) which determines the site of cleavage. Using two synthetic RNA oligomers, which can together form a hammerhead structure, it was demonstrated that cleavage can occur in trans, i.e. that one molecule can catalyze the cleavage of the other (Uhlenbeck, Nature 328 (1987), 596-600).

When cleavage occurs in trans, the RNA which can be cleaved is considered the substrate (target) RNA, and the RNA which catalyzes the cleavage is called an RNA enzyme or "ribozyme".
It is now possible to design an appropriate ribozyme against any GUC sequence motif of a given substrate RNA, so that cleavage occurs 3'-terminal of it (Haseloff and Gerlach, Nature 334 (1988), 585-591). Likewise GUA, GUU, CUA, CUC, AUC and UUC are suitable target sequences for the hammerhead-type ribozyme RNAs (Koizumi et al., FEBS Lett. 228 (1988),

228-230 and FEBS Lett. 239 (1988), 285- 288 and EP-A2 321 201). The motif GUG was cleaved in one case (Sheldon and Symons, Nucleic Acids Res. 17 (1989) 5679-5685), whereas it was not cleaved in others (Koizumi et al., FEBS Lett. 228 (1988) and Haseloff and Gerlach, Nature 334 (1988) 585-591). According to an investigation on the sequence requirements by Ruffner et al., Biochemistry 29, (1990) 10695-10702, any trinucleotide sequence NUH can be a target sequence (N can be A, C, G or U, H can be A,C or G). Thus, also the motifs AUA, AUU, CUU, UUA, UUU represent target sequences.

EP-A2 321 201 describes the ribozymes of the "hammerhead"-type and its usage to create RNA endonucleases which are, in contrast to the IVS ribozyme described in WO 88/04300, highly specific for a certain target RNA. In order to cleave a given substrate RNA (target RNA) at a certain site, a specific ribozyme RNA can be designed that consists of two functional regions: the actual catalytic domain, and the regions which are complementary to the target RNA, so that the ribozyme can bind to its substrate in a sequence-specific manner, and in that way that the catalytic domain of the ribozyme is placed opposite of the cleavage motif of the target RNA.

EP-A2 321 201 describes the procedure to create a ribozyme. For this purpose, a DNA oligonucleotide is synthesized according to a specific cleavage motif and the sequence context next to it within the target RNA. After cloning of this DNA oligonucleotide downstream of an appropriate promoter, this synthetic DNA can be used for the in vitro and in vivo synthesis of the target-specific ribozyme RNA. Transcription generates an RNA molecule which contains the catalytic ribozyme domain, flanked by sequences that are complementary to the target RNA and usually also vector-derived sequences which are not complementary to the target RNA. After the sequence-specific hammerhead conformation has been formed, cleavage occurs 3'-terminal to the motif of the substrate RNA, e.g. GUC. Since the base-pairing complementary regions between the substrate RNA and the ribozyme RNA which do not participate in the catalytic reaction, enable the sequence-specific binding of the ribozyme to its target, their length influences the specificity and efficiency of the ribozyme reaction.

The ribozymes of the hammerhead-type which are so far known from the approve prior art suffer from the disadvantage that for each and every use they have to be synthesized de novo. Such a synthesis is laborious, time consuming, and also involves technical difficulties because the synthetic ribozyme encoding DNA sequences have to have a length sufficient to warrant a reasonable preciseness of the cleavage of the target RNA. Therefore, so far there have been limits in the applicability of the ribozyme technology.

Accordingly, the technical problem underlying the present invention is to provide a means permitting the ease and precise construction of DNA sequences of sufficient length which encode ribozymes that cleave a desired target RNA.

The solution to the above technical problem is achieved by providing ribozyme cassettes consisting of DNA which can be inserted into selected restriction enzyme cleavage sites of the DNA sequence encoding said target RNA. Furthermore, it is achieved by providing the other embodiments characterized in the claims.

Thus, the present invention relates to portable ribozyme cassettes displaying the following features :

(a) a DNA sequence (a) encoding that part of a ribozyme which does not form base pairs with the target RNA; and

(b) DNA sequences (b) flanking the DNA sequence (a), which are derived from the protruding ends of a restriction enzyme cleavage site of a DNA sequence encoding a target RNA, and which encode a part of that part of a ribozyme which forms base pairs with the target RNA,

wherein after insertion of the ribozyme cassette into a target DNA that encodes the target RNA, said DNA sequences (a) and (b) encode together with that strand of said target DNA that is complementary to the target RNA (antisense strand), a ribozyme, having endoribonuclease activity for said target RNA.

The term "DNA sequence encoding that part of a ribozyme which does not form base pairs with the target RNA" refers to DNA sequences encoding the loop of ribozymes which may also be called the "active domain" or "catalytic domain" of the ribozyme. Examples of such a DNA sequence (a)

are the loops created when the ribozymes given in Figs. 1, 4, 7 and 13, pair with their target RNA (substrate RNA). Examples of a sequence (a) are also indicated in Figs. 2 and 3. When referred to a particular DNA strand, it is understood that the DNA strand that "encodes" the RNA is of the same polarity as the corresponding RNA. As shown in Fig.2, the sense strand of a cDNA sequence encodes the target RNA, whereas after insertion of the portable DNA cassette, the antisense strand, together with the sequence (a), encodes the ribozyme RNA that is directed against the target RNA.

The term "DNA sequences flanking the DNA sequence (a), which are derived from the protruding ends of a restriction enzyme cleavage site of a DNA sequence encoding a target RNA, and which encode a part of that part of a ribozyme which forms base pairs with the target RNA" refers to nucleotides neighboring the sequence (a). Therefore, sequences (b) do not represent a continuous sequence but consist of a part flanking sequence (a) at its 5'- end and another part at its 3'-end, respectively. Sequences (b) can also consist just of a part flanking the 5'-end of sequence (a) or just at the 3'-end, respectively. An example, of such DNA sequences (b) is given in Fig. 1, and 2.

In some embodiments of the present invention the portable ribozyme cassettes can be easily synthesized because - as is evident from the general formula presented hereinbelow - they only contain a few nucleotides. In other embodiments, the production of the portable ribozyme cassettes of the present invention preferably involves cloning steps because these portable ribozyme cassettes contain additional longer sequences, such as marker sequences.

The portable ribozyme cassettes of the present invention simplify the generation of target-specific ribozyme RNAs. For this purpose, they are inserted into the DNA encoding the target RNA, e.g. into a cDNA. As a result of this insertion, the cDNA encoding the target RNA is converted into a ribozyme construct, respectively, so that transcription can generate the desired catalytic antisense ribozyme RNA. As will be demonstrated below, more than 20 preferred restriction enzyme cleavage sites can be used for the insertion of the portable ribozyme cassettes of the present invention. A particular advantage of the ribozyme cassettes of the present invention is that it is not necessary to design a ribozyme RNA ac-

cording to the sequence of the target. In fact, it is not even necessary to know the nucleotide sequence of the target. A particular portable ribozyme cassette of the present invention, e.g. a SalI-specific cassette can be used in an universal way for the insertion into any SalI site in any target. The eventually resulting RNAs combine the characteristics of ribozymes and antisense RNAs and are therefore also called "catalytic antisense RNAs" or "antisense-ribozymes" or "antizymes". A striking advantage of the portable ribozyme cassettes of the present invention thus is their general applicability.

In a preferred embodiment, the present invention relates to a portable ribozyme cassette, wherein said DNA sequences (a) and (b) are represented by the following general formula:

$$\begin{array}{ll} 5' & M_2\ M_1\ K\ L\ T\ T\ C\ G^1(X)_x\ C^2Y_y\ T\ C\ W\ T\ C\ A\ G\ N_1\ N_2\ N_3\ N_4 \quad 3' \\ 3' & M'_2M'_1K'L'A\ A\ G\ C^1(X')_xG^2Y'_yA\ G\ W'A\ G\ T\ C\ N'_1N'_2N'_3N'_4 \quad 5' \end{array}$$

in which said DNA sequence (a) is represented by the sequence :

$$\begin{array}{lll} 5' & T\ T\ C\ G^1(X)_x\ C^2Y_y\ T\ C\ W\ T\ C\ A\ G & 3' \\ 3' & A\ A\ G\ C^1(X')_xG^2Y_y'A\ G\ W'A\ G\ T\ C & 5' \end{array}$$

and said DNA sequence (b) is represented by the sequences :

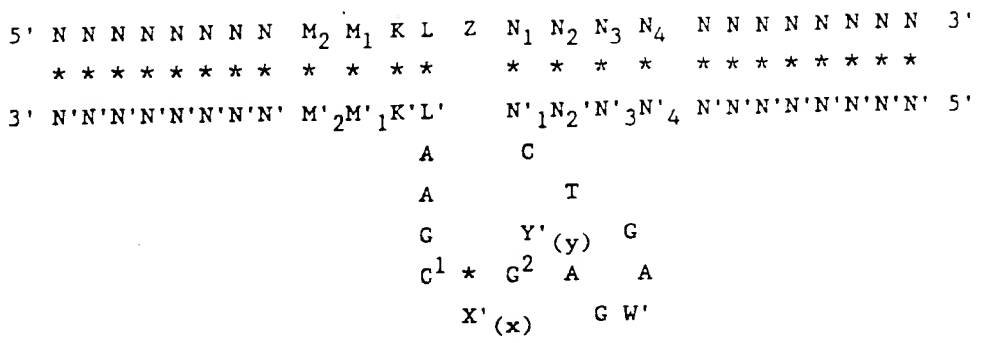
$$\begin{array}{lll} 5'\ M_2\ M_1\ K\ L & \text{and} & N_1\ N_2\ N_3\ N_4 \quad 3' \\ 3'\ M'_2M'_1K'L' & & N'_1N'_2N'_3N'_4 \quad 5' \end{array}$$

wherein:
the nucleotides K and L represent the first and second nucleotide (5'-3') of the target motif of the target RNA, and the nucleotides M_1 and N_1 are the first nucleotides flanking the target motif of the target RNA at the 5'- and 3'- side, respectively;
the nucleotides M_2 and N_2 are the second nucleotides flanking the target motif of the target RNA at the 5'- and 3'- side, respectively, and the nucleotides N_3 and N_4 are located in the third and fourth position at the 3'-side of the target motif of the target RNA;
the nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4, independently, are

A,G,C or T or not present in the cassette, under the proviso that
the presence of M_2 requires the presence of M_1,
the presence of M_1 requires the presence of K,
the presence of K requires the presence of L,
the presence of N_4 requires the presence of N_3,
the presence of N_3 requires the presence of N_2,
the presence of N_2 requires the presence of N_1,
and under the proviso that the total number of said nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4 is 0 to 4, preferably 1 to 4;
W, X or Y is A, G, C or T;
x is at least 6, wherein each X independently is A, G, C or T and wherein X is selected so that the complementary nucleotides X' form at least one additional base pair next to the base pair formed by the nucleotides C^1 and G^2 (which flank the sequence (X')) in the secondary structure which is formed when the ribozyme pairs to its target sequence;
y is 0 or 1;
when the nucleotide C^1 is C, the nucleotide G^1 is G, when the nucleotide C^1 is T, the nucleotide G^1 is A, when nucleotide C^1 is C or T, the nucleotides C^2 and G^2 are either C and G or T and A, respectively;
the nucleotides marked with " ' " are complementary nucleotides;
and
the nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4 correspond to nucleotides from the protruding ends obtained after cleavage of a restriction enzyme cleavage site within the DNA encoding the target RNA with the corresponding restriction enzyme, said restriction enzyme cleavage site containing in said protruding ends an additional nucleotide Z which is A,G,C or T.

The definitions given in context with the above general formula are given for the DNA level. As will be understood by the person skilled in the art, T is U when reference is made to RNA sequences.

The interaction between the target RNA and the ribozyme of the present invention can schematically be illustrated as follows:



wherein, the nucleotides N and N' are derived from the DNA encoding the target RNA,
the nucleotides K L Z represent the cleavage motif of the target RNA;
* represents a hydrogen bond;
the sequence 3' A A G $C^1(X')_{(x)}G^2Y'_{(y)}$A G W'A G T C 5' represents the sequence (a), that is the DNA sequence encoding that part of a ribozyme which does not form base pairs with the target RNA;
(x) is at least 6, wherein these X' residues form a loop structure with at least one additional base pair, preferably at least three base pairs, neighboring the C^1 * G^2 pair of the ribozyme within sequence (a); and
(y) is 0 or 1.

A specific example of such an interaction is depicted in Figure 1.

In this preferred embodiment of the present invention, the ribozyme cassette is inserted into a SalI site, wherein K is G, L is U, Z is C, N_1 is G, N_2 is A and N_3 is C, i.e. the nucleotides K, L, Z, N_1, N_2 and N_3 represent a SalI cleavage site contained in the DNA encoding the target RNA, wherein the nucleotides L, Z, N_1 and N_2 are located within the protruding ends created by SalI digestion. Simultaneously, the nucleotides L', N'_1 and N'_2 represent the nucleotides of the ribozyme cassette of the present invention which form base pairs with the target RNA on the RNA level. Again, it has to be understood that on the RNA level T is U. The latter part consisting of nucleotides L', N'_1 and N'_2 is also generally referred to as the sequence (b) of the present invention. Together with the loop depicted in Figure 1 (which is generally referred to as DNA sequence (a) in the present invention) and with the flanking nucleotides N' (including

the nucleotides M'_2, M'_1, K', and N'_3, N'_4, which fulfill in this particular sequence the same function as the nucleotides N'), given in the Figure 1, said DNA sequence forms the ribozyme ("antizyme") of the present invention.

It has to be understood that due to the polarity of the target RNA or the corresponding DNA sequence, the ribozyme of the present invention is depicted in 3'-5'-orientation in the claims, figures and illustrations of the present invention. Therefore, it will be evident to a person skilled in the art that a DNA sequence encoding the ribozyme or ribozyme cassette of the present invention has to be expressed in 5'-3'-orientation, i.e. in the opposite orientation as given in said figures and illustrations, in order to obtain the desired ribozyme as the expression product which has the desired endoribonuclease activity.

The superiority of the portable ribozyme cassettes of the present invention is evident from Figures 2 and 3. The portable ribozyme cassette is inserted into a given cleavage site of the DNA sequence encoding the target RNA (substrate) RNA, in this preferred embodiment a SalI site. The precise sequence specificity of the ribozyme ("antizyme") encoded by the resulting DNA sequence is automatically obtained without any difficult and time consuming steps or further manipulations. This is because the DNA sequence flanking the inserted ribozyme cassette is fully homologous to the DNA sequence encoding the target RNA.

Thus, according to the present invention, the length of the base pairing regions which influence the specificity and efficiency of the endoribonuclease activity of the ribozyme - because they are relevant for the correct and efficient binding of the resulting ribozyme to the target RNA - is only limited by the length of the DNA sequence into the restriction enzyme cleavage site of which the portable ribozyme cassette of the present invention has been incorporated. Thus, the present invention allows the construction of ribozymes, the base pairing region of which may have up to the same length as the substrate RNA if this is desired.

Depending on the meaning of the nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4 in the above general formula, this general formula either represents the <u>non-integrated</u> ribozyme cassette of the present invention ("portable

ribozyme cassette") or the integrated ribozyme cassette together with the total flanking sequences derived from the restriction enzyme cleavage site of the DNA sequence encoding the target RNA.
If the nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4 define the nucleotides from the protruding ends obtained after cleavage of said restriction enzyme cleavage site with the corresponding restriction enzyme, the above general formula represents the portable ribozyme cassette.
In cases where this portable ribozyme cassette lacks any of the above nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4, these have to be provided by the 5'-terminus of the DNA sequence encoding the target RNA in order to obtain a functional ribozyme.

In a preferred embodiment of the portable ribozyme cassette of the present invention, nucleotide L is T.

In a particularly preferred embodiments of the present invention, the nucleotides M_1, K, L, N_1, N_2, N_3 and N_4 have any of the following meanings:

	M_1	K	L	N_1	N_2	N_3	N_4
1	-	-	-	G	-	-	-
2	-	-	-	T	-	-	-
3	-	-	T	A	-	-	-
4	-	G	T	-	-	-	-
5	G	A	T	-	-	-	-
6	-	C	T	G	-	-	-
7	-	G	T	C	-	-	-
8	-	-	T	G	A	-	-
9	-	-	T	G	G	-	-
10	-	-	-	A	T	G	-
11	-	-	-	C	G	G	-
12	-	-	-	T	A	G	-
13	-	G	T	A	C	-	-
14	-	-	-	C	A	G	G
15	-	-	-	C	T	G	G
16	-	-	T	C	A	-	-
17	-	-	T	-	-	-	-

	M_1	K	L	N_1	N_2	N_3	N_4
18	-	-	-	A	-	-	-
19	-	-	T	A	A	-	-
20	-	T	T	A	-	-	-
21	A	A	T	-	-	-	-

In another preferred embodiment of the present invention, Y or W is selected such that it forms a restriction enzyme cleavage site together with the neighboring nucleotides.

The creation of such a restriction enzyme cleavage site within the portable ribozyme cassette of the present invention provides a means for screening for target DNA sequences in which the portable ribozyme cassette of the present invention has been incorporated. Furthermore, such a restriction enzyme cleavage site provides a means for the determination of the orientation in which the portable ribozyme cassette of the present invention has been incorporated into the target DNA sequence. Furthermore, such a restriction enzyme cleavage site provides a means for the excision of excess cassettes in case more than one cassette has been inserted into the DNA encoding the target RNA.

In another preferred embodiment of the present invention, X is selected in the above general formula such that it forms a restriction enzyme cleavage site either alone or together with the neighboring nucleotides. This restriction enzyme cleavage site also provides a means for screening and determination of the orientation of the portable ribozyme cassette of the present invention. In addition, this restriction enzyme cleavage site provides a means for the insertion of a marker sequence or any other desired sequence into the portable ribozyme cassette of the present invention. Thus, in a particularly preferred embodiment of the present invention, the portable ribozyme cassette also contains a marker sequence which is incorporated into the restriction enzyme cleavage site located at nucleotide X.

Surprisingly, it has been found, that the enzymatic activity of the ribozyme encoded by a desired DNA sequence into which the portable ribozyme cassette of the present invention has been incorporated is retained even after the insertion of marker sequences.

The marker sequence contained in said restriction enzyme cleavage site of the portable ribozyme cassette of the present invention allows a rapid selection of ribozyme constructs which contain the portable ribozyme cassette of the present invention. Therefore, particularly preferred marker sequences of the present invention represent a selectable marker gene, such as an antibiotic resistance gene or a gene such as the β-galactosidase gene, allowing the identification of desired recombinant DNA sequences by providing a color reaction.

In cases where it is not desired to retain the marker sequence in the portable ribozyme cassettes of the present invention, they can be removed by simply cleaving the constructed recombinant DNA sequences with the restriction enzyme recognizing the restriction enzyme cleavage site at position X and re-ligating the cleavage product, i.e. the DNA sequences flanking the originally contained marker sequence.

In a further preferred embodiment, the portable ribozyme cassettes of the present invention are cloned in a recombinant vector, so that said portable ribozyme cassette of the present invention is flanked by restriction enzyme cleavage sites. In a preferred embodiment these cleavage sites are derived from a class-IIS restriction enzyme that cleaves in a defined distance away from its actual recognition sequence that is located outside the sequence of the portable ribozyme cassette. This insertion of the portable ribozyme cassette of the present invention allows an excision and thus a simple transfer of the same into another target DNA sequence.
Moreover, the ribozyme cassette of the present invention which itself contains a restriction enzyme recognition sequence and which is cloned on a vector so that it can be precisely excised, may contain another DNA, e.g. a marker gene can be inserted. This enables the generation of a ribozyme cassette containing a marker gene.

In a further embodiment, the present invention relates to a DNA sequence encoding a ribozyme (antizyme), said DNA sequence containing a portable ribozyme cassette of the present invention in an orientation allowing the production of a ribozyme displaying endonuclease activity upon expression

in a host cell, or when used as template in a polymerase chain reaction, and containing additional sequences flanking said ribozyme cassette which have a length sufficient to provide a target-specific endoribonuclease activity of the encoded ribozyme.

These DNA sequences of the present invention can be used for the production of highly selective ribozymes (antisense ribozymes, antizymes) in the systems described below. They can be easily obtained by inserting the portable ribozyme cassette of the present invention into a restriction enzyme cleavage site of a DNA sequence encoding an RNA which is an antisense RNA in comparison to the target RNA. Therefore, as already mentioned, the ribozymes of the present invention are also called antisense ribozymes or "antizymes".

The additional sequences flanking the portable ribozyme cassette after insertion of it into a desired DNA sequence encode an antisense RNA in relation to the target RNA. These flanking sequences can be either a part of the antisense RNA or a full-length antisense RNA. Unless desired, a part of the antisense RNA having a length which is sufficient for highly selective binding of the ribozyme RNA to the target RNA can be used to express a target-specific ribozyme acting as endoribonuclease, e.g. in a given cell.

In a preferred embodiment of the present invention, said DNA sequences of the present invention which is contained in the recombinant vector is under the control of a suitable promoter. These recombinant vectors of the present invention are expression vectors which are suitable for the expression of the ribozymes of the present invention in a desired host cell. Examples of such procaryotic promoters are the bacteriophage lambda PL and PR promoters, the bacteriophage SP6, T3 and T7 promoters (which are preferred for the in vitro synthesis of the ribozymes of the present invention), the lacZ, tac, trc and the trp promoters. Examples for eucaryotic promoters in animal systems are the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR), the SV40 early and late promoters and for plant systems the nopaline synthase promoter, the cauliflower mosaic virus (CaMV) 35S promoter, and promoters derived from gemini viruses.

In a preferred embodiment of the present invention, the additional DNA sequences, which encode an antisense RNA, and which flank the portable ribozyme cassette after insertion, contain at their 5'- and/or their 3'-ends sequence domains that are self-complementary and that form after transcription into RNA highly stable stem-loop structures, which confer protection against exonucleases.

In a particularly preferred embodiment of the present invention, the sequences which after transcription into RNA form the stable stem-loop structure are derived from a double-stranded DNA cassette that can be inserted by blunt-end ligation and that has the following sequence:

5' pGCGGCCGCTCGGGCCACGCGAGGCCCGTGCGGCCGT 3'

3' CGCCGGCGAGCCCGGTGCGCTCCGGGCACGCCGGCAp 5' .

In a further embodiment, the present invention relates to recombinant vectors containing a portable ribozyme cassette of the present invention or the above mentioned DNA sequences of the present invention.

The present invention also relates to host organisms containing these recombinant expression vectors such as bacteria, e.g. E.coli or Bacillus subtilis, lower eucaryots, such as yeast or fungi.

In another embodiment, the present invention relates to a method for the production of a ribozyme of the present invention, said method comprising cultivating a host of the present invention under suitable conditions, and isolating the ribozyme of the present invention from the culture.

The present invention also relates to the ribozymes encoded by the DNA sequences of the present invention. Furthermore, the present invention relates to viral, bacterial, plant and animal genomes, containing a portable ribozyme cassette of the present invention or any other of the DNA sequences of the present invention referred to above. Additionally, the present invention relates to the corresponding viruses, bacteria, fungi, plants or animals containing such genomes. For instance, such viruses can be used as vectors for introducing DNA sequences encoding the ribozymes of the present invention into a desired host. Retroviruses and vaccinia viruses are preferred vector viruses.

As explained herein above, the transcription of the DNA sequences encoding the ribozymes of the present invention gives rise to ribozymes which act to inactivate a desired target RNA (substrate RNA). Thus, either the DNA sequences encoding the ribozymes of the present invention or the ribozymes themselves have extensive therapeutic and biological applications. For example, they can be used for the suppression of certain unwanted genes or the treatment of viral infections in man, animals and plants by inactivating a target RNA, produced in the life cycle of the virus. Thus, the ribozymes of the present invention can be used for the treatment of infections with retroviruses such as the human immunodeficiency virus (HIV) and positive-sense RNA viruses, such as infections with togaviruses, coronaviruses, picornaviruses, caliviviruses and also for infections with negative-sense RNA viruses such as paramyxoviruses (e.g. Sendai virus), rhabdoviruses, the influenca viruses, the bunyaviruses, or arenaviruses.

Also the viral mRNAs of DNA viruses can be targeted by ribozymes of the present invention, so that they can be used for treatment of viral infections with pox-, irido-, herpes- (e.g herpex simplex virus (HSV)), adeno, papova- (e.g. hepatitis B virus (HBV) and/or reoviruses. The ribozymes and the corresponding encoding DNA sequences of the present invention can also be used for the inactivation of target RNAs in procaryotic cells, such as bacteria, or eucaryotic cells such as protozoa and yeast, in plants and animals, such as parasitic animals, e.g. plasmodium faliparum, and humans. In the treatment of humans, the ribozymes of the present invention or the corresponding encoding DNA sequences may be administered to a patient in need thereof.

Therefore, the present invention also relates to a composition containing a ribozyme or a corresponding encoding DNA sequence of the present invention. The present invention furthermore relates to a method of eliminating a target RNA in a plant, an animal or a human patient, which comprises treating said plant animal or human patient with a DNA sequence encoding the ribozyme of the present invention or with a ribozyme of the present invention, optionally in association with a pharmaceutically, veterinarially or agriculturally acceptable carrier and/or excipient. By using such compositions, the ribozymes or the corresponding encoding DNA sequences of the present invention may be delivered by parenteral or

other means of administration.

In a preferred embodiment of the compositions of the present invention, the DNA sequence encoding the ribozyme of the present invention and containing a portable ribozyme cassette of the present invention is contained in a vehicle, such as a carrier virus, by which it is transported to a particular target tissue or cell into the genome of which it can be incorporated or in which it can be transiently expressed. A carrier virus which may be used here for example is a recombinant retrovirus or a recombinant vaccinia virus.

In the case of plants, the compositions of the present invention may contain Ti-plasmid based vectors or vector systems or correspondingly transformed Agrobacteria, which are capable of directing the transfer of the DNA sequences of the present invention containing the portable ribozyme cassette into a desired target tissue or target cell of the plant to be treated. Alternatively, the compositions of the present invention may contain expression vectors which are suitable for direct gene transfer techniques into plant cells or tissues, such as electroporation or particle acceleration.
Again, the expression of the ribozyme in such a plant target tissue or target cell may be either transient or permanent.

Thus, the present invention also relates to compositions for eliminating the disease-causing capability of an infectious agent.

The present invention also relates to a method for the production of a DNA sequence encoding a ribozyme, wherein this DNA sequence contains a portable ribozyme cassette as described herein, said method comprising the steps of:

(A) selecting in a DNA sequence encoding a desired target RNA, which is to be inactivated by a ribozyme, a restriction enzyme cleavage site of the following nucleotide sequence:
$M_2\ M_1\ K\ L\ Z\ N_1\ N_2\ N_3\ N_4$,
wherein M_2, M_1, K, L, N_1, N_2, N_3, and N_4, have the same meaning as given before;

Z is A, G, C or T;
Z corresponds to the 3'-terminal nucleotide of the 5'-terminal ribozyme cleavage product of the target RNA;
the nucleotide sequence K L Z corresponds to a nucleotide sequence of the target RNA which is cleavable by a ribozyme;
and wherein Z is part of the protruding ends obtained after cleavage of said restriction enzyme cleavage site with the corresponding restriction enzyme;

(B) cleaving said restriction enzyme cleavage site of the DNA sequence given in (A) with the corresponding restriction enzyme;

(C) removing the protruding ends of the cleavage product of (B) and creating blunt ends;

(D) producing a ribozyme cassette by carrying out a method comprising the following steps :

(DA) adding to the 5'-terminus of a DNA sequence (a), as defined in the preceding claims, the nucleotides located at the 5'-side of Z in the protruding ends as obtained in (B); and

(DB) adding to the 3'-terminus of said DNA sequence (a) the nucleotides located at the 3'-side of Z in said protruding ends;

(E) insertion of the ribozyme cassette obtained in step (D) into the DNA sequence obtained in step (C).

In a preferred embodiment of this method, the ribozyme cassette displaying the structure given in step (D), which is inserted in step (E) into the DNA sequence obtained in step (C), is excised from a cloning vector by cleavage of restriction enzyme cleavage sites flanking said portable ribozyme cassette.

Furthermore, the present invention relates to a method for the production of a portable ribozyme cassette of the present invention comprising the steps of :

(A) selecting in a DNA sequence encoding a desired target RNA, which is to be inactivated by a ribozyme, a restriction enzyme cleavage site of the following nucleotide sequence:

$M_2\ M_1\ K\ L\ Z\ N_1\ N_2\ N_3\ N_4$,

wherein M_2, M_1, K, L, N_1, N_2, N_3, and N_4, have the same meaning as before;
Z is A, G, C or T;
Z corresponds to the 3'-terminal nucleotide of the 5'-terminal ribozyme cleavage product of the target RNA;
the nucleotide sequence K L Z corresponds to a nucleotide sequence of the target RNA which is cleavable by a ribozyme;
and wherein Z is part of the protruding ends obtained after cleavage of said restriction enzyme cleavage site with the corresponding restriction enzyme;

(B) determining the protruding ends of said restriction enzyme cleavage site which are created by cleaving with the corresponding restriction enzyme; and

(C) producing a ribozyme cassette by carrying out a method comprising the following steps :

(CA) adding to the 5'-terminus of a DNA sequence (a), as defined in the preceding claims, the nucleotides located at the 5'-side of Z in the protruding ends determined in (B); and

(CB) adding to the 3'-terminus of said DNA sequence (a) the nucleotides located at the 3'-side of Z in said protruding ends.

In a preferred embodiment of the present invention, the technique to generate a DNA construct that encodes an antisense-ribozyme, (which is directed against a particular target RNA (substrate RNA)), comprises five steps: (i) the preparation of the DNA cassette, (ii) the preparation of the cDNA that codes for the target RNA (substrate RNA), (iii) the actual insertion of the DNA cassette into the cDNA, (iv) the analysis of the recombinant clones and (v) the confirmation of the catalytic activity of

the resulting RNA in a ribozyme assay. The individual phases of the procedure are done as follows. A more detailed description of some conventional laboratory procedures are found in Sambrook et al., "Molecular Cloning", Cold Spring Harbor, second edition, 1989.

(i) Preparation of the DNA cassette:

As outlined in Example 2 and Fig.8, for each desirable DNA cassette an oligodeoxynucleotide is cloned in a plasmid vector, which is preferably the plasmid pT3T71ac (Boehringer Mannheim, FRG). The resulting recombinant plasmid (for example plasmid pAzSal1) contains a DNA sequence, corresponding to the desired DNA cassette between two recognition sites of a class-IIS restriction enzyme, like EarI, that allows to specifically release a DNA fragment with defined ends so that after filling-in of its protruding ends the desired DNA cassette can be obtained. After confirming the correct cloning of the oligodeoxynucleotide by sequence analysis, the plasmid that carries the ribozyme DNA cassette is cleaved with XhoI. A DNA fragment that is flanked by XhoI sites and that contains the tetracycline resistance gene (tet gene) is inserted. This DNA fragment is obtained from a modified version of the plasmid pBR322 that had been generated by successive introduction of XhoI linkers into the EcoRI and AvaI sites of plasmid pBR322, respectively, after cleaving with said restriction enzymes and subsequent filling-in with Klenow polymerase (the EcoRI site is thus restored). Then, the orientation in which the tet gene had been inserted into the XhoI site is determined by digestion with EcoRI and HindIII, which cleave once within the tet gene and once in the plasmid pT3T71ac. After completing this analysis, the actual DNA cassette is generated by cleaving the recombinant plasmid (for example pAzSal1-tet) with EarI. About 5-10 µg of the plasmid are digested with about 20-30 units of the isoschizomer Ksp632I from Boehringer Mannheim in a volume of 100 µl. After 2-3 hours at 37°C, dNTPs are added to a final concentration of 500 µM, 5 units of the large fragment of E.coli DNA polymerase I (Klenow enzyme, N.E. Biolabs) are added and the reaction mixture is incubated for 5 minutes at 16°C. After addition of EDTA to a final concentration of 15 mM, the DNA is loaded in several lanes on an agarose gel. After separation of the DNA fragments by electrophoresis, the gel is stained with ethidium bromide (1 µg/ml) and the fragment of about 1450

base pairs, corresponding to the desired DNA cassette, is excised. The DNA is electroeluted, phenolized and collected by isopropanol precipitation. The DNA cassette is then dissolved in a small volume (10-20 µl) of TE buffer (10 mM Tris/HCl, 1 mM EDTA pH 8.0). A small aliquot (1 µl) is again loaded on an agarose gel to estimate the concentration of the recovered DNA cassette.
The residual solution of the DNA cassette is stored at -20°C and can be used for several incorporation experiments.

(ii) Preparation of the cDNA

The cDNA (or a fragment of it) that encodes the target RNA is cloned into a cloning vector that allows in vitro transcription of the inserted DNA in different directions from bacteriophage-encoded RNA polymerase promoters, such as T3, T7 or SP6 RNA polymerase. Many of these "gemini"-type vectors are commercially available. A preferred example is the transcription vector pT3T7lac (or modifications thereof, where one restriction site had been destroyed) from Boehringer, Mannheim, FRG. The plasmid contains a polylinker region between the promoters for T7 and T3 RNA polymerase. This enables the in vitro synthesis of RNA that corresponds to the substrate RNA or its complementary RNA (compare Fig.2). Thus, labeled RNA can be synthesized that can act as substrate for the ribozyme assay (see below).
After selection of a restriction site where a corresponding DNA ribozyme cassette should become introduced, one µg of the recombinant plasmid is cleaved with said restriction enzyme. After phenolizing and subsequent collection of the plasmid by isopropanol precipitation, that plasmid is dissolved in 18 µl of S1 buffer (225 mM NaCl, 30 mM potassium acetate pH 4.5, 200 µM $ZnSO_4$ and 5% glycerol) and incubated in ice for at least 10 minutes to be cooled down to 0°C. In parallel, nuclease S1 (Boehringer Mannheim) is diluted in S1 buffer to a concentration of 1 unit per µl and also incubated for at least 10 minutes on ice. Then, 2 µl, corresponding to two units of nuclease S1, are added to the cleaved plasmid. After 20 minutes on ice, 5 µl of S1 Stop buffer (300 mM Tris/HCl, 50 mM EDTA, pH 8.0) are added to the reaction mixture, which is in the following incubated for 10 minutes at 65°C for inactivation of the nuclease. The sample is subsequently phenolized and again precipitated with isopropanol.

It is dissolved in a small volume of TE buffer (10 µl) and 1 µl is loaded on an agarose gel to estimate the concentration.

(iii) Insertion of the DNA cassette into the cDNA:

About 200 - 300 ng of the cDNA-containing plasmid, cleaved with the restriction enzyme, into which the DNA ribozyme cassette should be introduced, and the resulting protruding ends trimmed by treatment with nuclease S1 is mixed with about 100 ng of the DNA ribozyme cassette and ligated in a small volume (5 - 10 µl) of blunt end ligation buffer (50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 5% PEG 8000, 1 mM DTT and 100 µM ATP with 3 units of T4 DNA ligase (Minotech, Heraklion, Greece) for at least 12 hours at 12°C. The ligation mixture is then transformed into E.coli, preferably strain JM83, and plated on agar plates that contain 13 µg/ml tetracycline and 100 µg/ml ampicillin.

(iv) Analysis of the recombinant clones

After isolation of plasmids from the recombinant clones (minipreps), "restriction analysis" is carried out, in order to test for the presence of the StuI site originating from the DNA cassette. XhoI digestion confirms that the tet gene can be excised. The orientation of insertion is determined by using a restriction enzyme that cleaves within the tet gene and in the cDNA insert or within the vector, e.g. BamHI, EcoRI or HindIII. Depending on the orientation of insertion of the tet gene, it can be concluded whether a sense- or an antisense directed- ribozyme has been constructed.

(v) Ribozyme assay:

The correct generation of the ribozyme RNA is confirmed by a functional test. For this purpose, labeled substrate RNA is prepared by in vitro transcription, using the transcription vector into which the cDNA fragment of interest had been cloned. The ribozyme constructs are cleaved with a restriction enzyme (which does not cleave within the tet gene, nor within the cDNA insert), so that they provide a template for run-off transcription. The ribozyme RNA is prepared by in vitro transcription

(unlabeled) from the corresponding promoter under standard reaction conditions (according to the supplier of the enzymes (N.E. Biolabs or Boehringer Mannheim) in 20 µl for one hour at 37°C. In the last two minutes of the reaction, 4 units of DNaseI, RNase-free (Boehringer Mannheim) are added. Then the mixture is phenolized and precipitated with ethanol. The ribozyme RNA is then incubated with the labeled substrate RNA in 20 µl of 50 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$ for 30 minutes at 60°C. After addition of sodium acetate (pH 5) to a final concentration of 0.2 M and 1 µg of tRNA (used as "carrier"), the samples are precipitated with ethanol and separated on a denaturating 5% polyacrylamide gel as described by Tsagris et al., EMBO J., 6 (1987), 2173-2183, and visualized by autoradiography. Detection of cleavage products of expected size confirm the ribozyme nature of the tested RNAs.

Ultimately, the corresponding DNA construct is cleaved with XhoI and religated in order to excise the tet gene.

For a fast analysis, the ribozyme RNA can also be taken directly after transcription (without phenolizing and precipitation the ribozyme RNA). In that case, 20 µl of labeled substrate RNA in 100 mM Tris/HCl pH 8.0 and 40 mM $MgCl_2$ are added to the unlabeled ribozyme RNA and the reaction mixture is incubated for 30 minutes at 60°C. Then 20 µl of 0.6 M sodium acetate, 150 µg/µl tRNA is added, followed by 150 µl of ethanol. The samples are collected by centrifugation and separated on a denaturating polyacrylamide gel together with an aliquot of labeled but untreated substrate RNA. After visualization by autoradiography, cleavage products of the substrate can be identified. Due to the lack of phenolization of the ribozyme RNA, the substrate RNA might undergo some unspecific degradation. The cleavage products can, however, be identified. Thus, the ribozyme activity of certain constructs can be verified.

Examples of restriction enzyme cleavage sites (taken from the catalogue of New England Biolabs) which can be used in the above mentioned methods of the present invention are listed in the following Table I.

Table I

Examples of restriction enzyme cleavage sites for the insertion of restriction enzyme-specific portable ribozyme cassettes[a]

Restriction[b] enzyme	Target[c] motif KLZ	No.of cases[d] in %	sequence after trimming[e]		DNA cassette[f] $M_1K\ L\ (a)\ N_1N_2N_3N_4$
ATC motifs:					
BamHI	-G'GATCC-	100	-G	C-	G A T (a) - - - -
BclI	-T'GATCA-	100	-T	A -	G A T (a) - - - -
BglII	-A'GATCT-	100	-A	T -	G A T (a) - - - -
BstYI	-RGATC'Y-	100	-R	Y -	G A T (a) - - - -
ClaI	-AT'CGAT-	100	-AT	AT-	- - - (a) G - - -
CTA motifs:					
AvrII	-C'CTAGG-	100	-C	G-	- C T (a) G - - -
NheI	-G'CTAGC-	100	-G	C-	- C T (a) G - - -
SpeI	-A'CTAGT-	100	-A	T-	- C T (a) G - - -
XbaI[g]	-T'CTAGA-	100	-T	A-	- C T (a) G - - -
CTC motifs:					
XhoI	-C'TCGAG-	100	-C	G-	- - T (a) G A - -
AvaI	-C'YCGRG-	50	-C	G-	- - T (a) G R - -
CTN motifs[i]:					
Bsu36I	-CC'TNAGG-	50	-CC	GG -	- - T (a) A - - -
DdeI	-C'TNAG-	50	-C	G -	- - T (a) A - - -
EspI	-GC'TNAGC-	50	-GC	GC -	- - T (a) A - - -

Continuation of Table I

Restriction[b] enzyme	Target[c] motif KLZ	No.of cases[d] in %	sequence after trimming[e]		DNA cassette[f] M_1K L (a) $N_1N_2N_3N_4$
GTA motifs :					
Asp718	-G'GTACC-	100	-G	C-	- G T (a) C - - -
KpnI	-GGTAC'C-	100	-G	C-	- G T (a) C - - -
AccI[h]	-GT'ATAC-	50	-GT	AC-	- - - (a) T - - -
GTC motifs :					
SalI	-G'TCGAC-	100	-G	C-	- - T (a) G A - -
AccI[h]	-GT'CGAC-	50	-GT	AC-	- - - (a) G - - -
AvaII	-G'GWCC-	50	-G	C-	- G T (a) - - - -
PpmuI	-RG'GWCCY-	50	-RG	CY-	- G T (a) - - - -
RsrII	-CG'GWCCG-	50	-CG	CG-	- G T (a) - - - -
EcoO109I	-RG'GNCCY-	25	-RG	CY-	- G T (a) - - - -
GTN motifs:					
BstEII	-G'GTNACC-	100	-G	C-	- G T (a) A C - -
MaeIII	-'GTNAC-	100	-	-	- G T (a) A C - -
GTG motif:					
ApaLI	-G'TGCAC-	100	-G	C-	T (a) C A - -
NTC motifs:					
BspHI	-(N)T'CATGA-	100	-(N)T	A-	- - - (a) A T G -
BspMII	-(N)T'CCGGA-	100	-(N)T	A-	- - - (a) C G G -
TaqI	-(N)T'CGA-	100	-(N)T	A-	- - - (a) G - - -
XbaI[g]	-(N)T'CTAGA-	100	-(N)T	A-	- - - (a) T A G -
EcorII	(NT)'CCWGG-	25	-(NT)	-	- - - (a) C W G G

Continuation of Table I

Restriction[b] enzyme	Target[c] motif KLZ	No.of cases[d] in %	sequence after trimming[e]	DNA cassette[f] M_1K L (a) $N_1N_2N_3N_4$
TTC motif:				
BstBI	-T'TCGAA-	100	-T A-	- - T (a) G A - -
ATA motif:				
NdeI	-CA'TATG-	100	-CA TG-	- - T (a) - - - -
ATT motifs:				
AseI[k]	-AT'TAAT-	100	-AT AT-	- - - (a) A - - -
EcoRI	-G'AATTC-	100	-G C-	A A T (a) - - - -
CTT motif:				
AflII[l]	-C'TTAAG-	100	-C G-	- - T (a) A A - -
TTA motifs:				
AseI[k]	-AT'TAAT-	100	-AT AT-	- - T (a) - - - -
AflII[l]	-C'TTAAG-	100	-C G-	- T T (a) A - - -

Foot notes :

a: A detailed example for the interpretation of the Table is given below;

b: The restriction enzymes are listed which can be used for insertion of a ribozyme cassette; with the exception of TaqI, only restriction enzymes with a recognition sequence of five and more nucleotides are listed.

c: The recognition sequences of the restriction enzymes, which is part of the DNA encoding the target RNA, are given,
the cleavage site is indicated by "'",
the nucleotides N are A, C, G or T, W are A or T, Y are C or T, R are A or G,
the target motifs consisting of the three nucleotides KLZ (as defined before) within the restriction sites are underlined,
all the restriction enzymes listed produce such protruding ends that they contain the nucleotide Z in it.

d: The percentage gives the proportion of restriction sites which provide a target motif - e.g. the sequence GGTCC represents only approximately 50 % of all possible AvaII sites with the recognition sequence GGWCC. Only 50% of the AvaII sites can be expected to be target motifs.

e: Remaining nucleotides of the restriction recognition sequence after digestion and removing the protruding ends.

f: The sequence of the DNA cassette which is used for insertion into the DNA encoding a target RNA, for creating a ribozyme as described. The DNA cassette consists of the sequence (a), as defined before, to which some nucleotides out of M_1, K, L and N_1, N2, N_3, and N_4, also defined before, are added 5'-terminal and 3'-terminal to sequence (a), respectively.

g: XbaI contains two target motifs: -(N)T'CTAGA- and -T'CTAGA-.

h: There are two possible AccI sites GT'CGAC and GT'ATAC which contain a GTA and GTC motif, respectively.

i: So far, cleavage has only been observed for CTA and CTC motifs.

k: AseI contains two target motifs: AT'TAAT and AT'TAAT.

l: AflII contains two target motifs: C'TTAAG and C'TTAAG.

For the better understanding of the present invention, in the following an example of how to apply and interpret Table I is given:

If, for instance, a ribozyme is desired which contains a GTC target motif within a SalI recognition sequence, the DNA cassette is selected according to Table I as follows:

The SalI site is

-GTCGAC- (in Table I given as G'TCGAC)
-CAGCTG-

After digestion :

-G TCGAC-
-CAGCT G-

and trimming :

-G C- (in Table I given as -G C-)
-C G-

The portable ribozyme cassette of the present invention with the catalytic domain which has to be inserted may for instance consist of the following core sequence (DNA sequence (a)), and the "replacement nucleotides" which are the DNA sequence (b) (here, 5'- end: L is T, and 3'-end: N_1 is G and N_2 is A) which are specific for each restriction recognition sequence:

5' T TTCGGCCTCGAGGCCTCATCAG GA 3'
3' A AAGCCGGAGCTCCGGAGTAGTC CT 5'
core sequence
(DNA sequence (a))

5' 3'
(in Table I given as : T (a) GA)

In order to create a ribozyme of the present invention which is specific for a particular target RNA, only two requirements must be fulfilled :

(i) at least a partial DNA construct encoding the target RNA (substrate RNA) must be available, preferably cloned in a plasmid or phage vector (sequence information is not necessary !), and

(ii) the DNA construct must contain a restriction enzyme cleavage site as defined hereinabove in the general formula, e.g. a restriction enzyme cleavage site as given in Table I.
All these sequence motifs contain one of the cleavage motifs required for ribozyme activity.

The sequences of portable ribozyme cassettes which are preferred on the basis of our current knowledge on restriction enzyme cleavage sites and on the possible target motifs are summarized in Table II.

Table II

Summary of the different DNA cassettes used for insertion into different restriction sites in order to create a DNA that encodes for a ribozyme

No.	sequence of DNA cassette[a]	restriction enzyme(s)[b]	nucleotides present[c] in DNA cassette	sum of nucleotides in sequence (b)[d]
1	(a) G	AccI[e] ClaI TaqI	N_1	1
2	(a) T	AccI[e]	N_1	1
3	T (a) A	Bsu36I DdeI EspI	L N_1	2
4	G T (a)	AvaII Eco0109I PpmuI RsrII	K L	2
5	G A T (a)	BamHI BclI Bgl II BstyI	M_1 K L	3
6	C T (a) G	AvrII NheI SpeI XbaI[f]	K L N_1	3
7	G T (a) C	Asp718 KpnI	K L N_1	3

Continuation of Table II

No.	sequence of DNA cassette[a]	restriction enzyme(s)[b]	nucleotides present[c] in DNA cassette	sum of nucleotides in sequence (b)[d]
8	T (a) G A	AvaI[e] BstBI SalI XhoI	L N_1 N_2	3
9	T (a) G G	AvaI[e]	L N_1 N_2	3
10	(a) A T G	BspHI	N_1 N_2 N_3	3
11	(a) C G G	BspMII	N_1 N_2 N_3	3
12	(a) T A G	XbaI[f]	N_1 N_2 N_3	3
13	G T (a) A C	BstEII MaeIII	K L N_1 N_2	4
14	(a) C A G G	EcoRII[e]	N_1 N_2 N_3 N_4	4
15	(a) C T G G	EcoRII[e]	N_1 N_2 N_3 N_4	4
16	T (a) C A	ApaLI	L N_1 N_2	3
17	T (a)	NdeI AseI[g]	L	1
18	(a) A	AseI[g]	N_1	1
19	T (a) A A	AflII[h]	L N_1 N_2	3
20	T T (a) A	AflII[h]	K L N_1	3
21	A A T (a)	EcoRI	M_1 K L	3

Foot notes

a : The DNA cassette consists of sequence (a) as defined plus the additional nucleotides as indicated;

b : The restriction enzymes which can be used in combination with this DNA cassette;

c : The nucleotides added to sequence (a) of the DNA cassette given in the general form as defined before;

d : indicates the sum of the nucleotides added to sequence (a);

e : These restriction enzymes can be used in combination with two different DNA cassettes depending on the recognition sequence of that particular cleavage site.

f : Since XbaI contains the two target motifs: -(N)T'CTAGA- and -T'CTAGA, every XbaI site can be used with each of the two DNA cassettes.

g : AseI contains two target sites: AT'TAAT and AT'TAAT.

h : AflII contains two target sites: C'TTAAG and C'TTAAG.

The Figures show :

Fig 1.

The hammerhead conformation between a substrate RNA (target RNA) containing a SalI recognition sequence (GUCGAC) and its corresponding ribozyme RNA.
The part of the ribozyme molecule which is bulged out is the catalytic domain and residues which are assumed to participate in the cleavage reaction are boxed. The site of cleavage is indicated.
In this particular case, the sequence GUC is the target motif of the substrate RNA. Therefore, these nucleotides represent the nucleotides KLZ, wherein nucleotide Z (in this case C) is the only nucleotide of the substrate RNA that does not directly base pair with the ribozyme RNA and which is that residue, that corresponds to the 3'-terminal nucleotide of the 5'-terminal ribozyme cleavage product of the target RNA as indicated.
The sequence 5' CUGAUGAGUCCGUGAGGACGAA 3' represents a sequence (a), i.e. that part of a ribozyme which does not form base pairs with the target

RNA.

After cleavage of a DNA encoding the substrate RNA with SalI, the nucleotides UCGA of the target RNA are located within the protruding ends. The nucleotides U and GA of the substrate RNA and A and CU of the ribozyme RNA are part of the protruding ends of the SalI site and are also part of the base pairing region between substrate RNA and ribozyme. Therefore, they represent the sequence (b), i.e. the DNA sequences flanking the sequence (a) which are derived from the protruding ends of a restriction enzyme cleavage site of a DNA sequence encoding a target RNA, and which encode a part of that part of a ribozyme which forms base pairs with the target RNA.

Fig.2

The cDNAs which allow transcription to yield the target RNA and the ribozyme RNA.

The top shows the cDNA of a target RNA with a SalI site as indicated, and the lower part represents the corresponding ribozyme DNA construct. Upon transcription in the directions indicated by arrows, the target and the ribozyme RNA (which can be considered as an antisense RNA with a ribozyme domain) will be synthesized which can form the hammerhead structure as given in Fig.1. The sense strand of the cDNA encodes the target RNA and the antisense strand of the corresponding ribozyme construct encodes the ribozyme RNA. The sequence of the two DNAs differ only in the two parts which are boxed, wherein the lower box represents sequence (a). The base pairs T/A and GA/CT which neighbor the boxed C/G pair in the target RNA and the boxed sequence (a) in the antisense ribozyme construct, represent the sequence (b) derived from the protruding ends of the SalI cleavage site.

This figure summarizes the principle of the present invention in which the boxed C/G pair within the DNA encoding the target RNA is replaced by the box given at the bottom, resulting in a DNA construct which gives rise to a ribozyme RNA specific for the target RNA when an antisense (antizyme) RNA is synthesized utilizing the DNA construct as template.

Fig.3

Strategy for creating a ribozyme construct directed against an RNA with a SalI recognition sequence.
The top illustrates a double-stranded cDNA containing a SalI site with the sense strand at the top. The four internal nucleotides TCGA are removed by digestion with SalI and subsequent trimming of the protruding ends. By insertion of a synthetic SalI-specific double-stranded DNA cassette, a sense- or antisense-directed ribozyme construct can be obtained, depending on the orientation of insertion. The hatched part of the synthetic DNA cassette corresponds to the lower strand of the boxed sequence of Fig.2 and represents a sequence (a) within the ribozyme sequence. In this case, the sequence (a) is modified to contain a StuI site (AGG'CCT) as given in Fig.4. The nucleotides which are attached (5'T and 3'GA, and 5' TC and 3'C, respectively) comprise sequence (b) and correspond to nucleotides L, N_1 and N_2 of the general formula. They are used to replace three of the four nucleotides which have been removed by SalI digestion and subsequent trimming.
The nucleotide C in third position of the SalI site corresponds to nucleotide Z, and is not replaced by inserting the cassette.

Fig.4

The hammerhead structure formed by a ribozyme RNA that contains a StuI recognition sequence and is directed against an RNA containing a SalI recognition sequence.
The substrate RNA contains a SalI recognition sequence like in Fig.1. The catalytic domain is modified by three nucleotide exchanges. The newly introduced residues G,U,C (5'->3') are given in bigger letters and the original nucleotides are indicated in parenthesis. The nucleotide exchanges create a new StuI recognition sequence AGGCCU and opposite of it an additional HaeIII sequence GGCC.

Fig.5

Map of the plasmids pPV1 and pPV2 and their resulting antizyme constructs.
The two maps at the top show the plasmids pPV1 and pPV2 which each contain an EcoRI fragment of the cDNA of plum pox virus (PPV) ranging from nucleotide 3409-3831, with a SalI site at nucleotide 3631, which is detailed. The EcoRI fragment is inserted in different orientations in the two plasmids. The right legend indicates the polarity of the PPV RNA which can be synthesized with T3 and T7 RNA polymerase from these plasmids, respectively.
The two maps in the middle are antizyme constructs derived from plasmid pPV1 and the two maps at the bottom represent derivatives of plasmid pPV2. The hatched parts of the DNA cassette in the four antizyme constructs, represent the sequence (a) of the ribozyme sequence. The right legend indicates whether T3 or T7 RNA polymerase can synthesize a sense or an antisense-directed ribozyme construct.

Fig.6

Autoradiograph of the gelelectrophoretic analysis for cleavage of PPV target RNA by an antizyme RNA.
The plasmids pPV2 and pPV12 were used to synthesize radioactively labeled PPV (-) RNA serving as substrate (S) and antizyme RNA (Az), respectively, by transcription with T7 RNA polymerase. The RNAs were incubated in 50 mM Tris/HCl, 20 mM $MgCl_2$, pH 8.0 for 30 minutes at either 0°C or 60°C, alone or in mixture. After collection by ethanol precipitation, the RNAs were analyzed on an polyacrylamide gel (5% polyacrylamide, 0.125% bisacrylamide) containing 8M urea.
Lane 1, antizyme RNA (Az) from pPV12, incubated at 0°C, Lane 2 PPV substrate RNA (S) from plasmid pPV2, incubated at 0°C, Lanes 3 and 4 correspond to 1 and 2, but the incubation temperature is 60°C. Lane 5 contains both RNAs of lane 3 and 4 incubated together. Lane 6 is like in Lane 5, but the ribozyme RNA is not radioactively labeled (indicated by AZ in parenthesis). Lane 7 contains a truncated marker transcript (T) synthesized by T7 RNA polymerase from plasmid pPV2 which had been cleaved with

SalI before, so that it corresponds to the expected 5'-terminal cleavage product.
As can be seen in Lanes 5 and 6, the PPV target RNA is cleaved into two smaller RNAs by the antizyme RNA. The smaller of the two cleavage products co-migrates with the marker transcript in lane 7 and represents the 5'-half of the PPV target transcript.
After a reaction time of 30 minutes, more than half of the substrate RNA was cleaved.

Fig.7

The hammerhead structure with a ribozyme RNA, specific for a SalI recognition sequence, that contains an XhoI and a StuI recognition sequence.
Compared to the structure in Fig.4, an additional base modification (U to C) is made, creating now an XhoI site (CUCGAG) that forms a hairpin loop of the catalytic domain.

Fig.8

Strategy for constructing an universal SalI-ribozyme-cassette with selection for the tetracycline resistance gene (tet gene).
The sequence at the top represents a part of the plasmid pAzSall. After digestion with the restriction endonuclease EarI and subsequent filling-in of the protruding ends, a 25 bp DNA cassette is formed which can be used to create ribozyme constructs as described in Fig.3.
By inserting the tetracycline resistance gene (tet gene) into the XhoI site of plasmid pAzSall, the plasmid pAzSall-tet is obtained (lower part). EarI digestion followed by Klenow reaction results in a cassette as shown at the bottom, which can equally be used for insertion into a trimmed SalI site. Using tetracycline, it can be selected for the insertion of the portable ribozyme cassette.

Fig.9

Schematic map of different types of antisense ribozyme RNAs.
The peculiarity of antisense ribozymes is that they contain a long region of sequences complementary (antisense) to the target RNA. Antizymes with one or two catalytic domains are given in the two upper maps. The map at the bottom represents an antizyme RNA, in which a foreign RNA, e.g. the mRNA of the tetracycline resistance gene, has been introduced. It should be stressed that in case of the tetracycline resistance gene, the inserted sequence is more than 50-fold bigger as compared to the actual catalytic domain.

Fig.10

Map of plasmid pPV12-tet.
Plasmid pPV12-tet was created from plasmid pPV1 (Fig.5) by inserting the SalI-specific DNA cassette obtained from plasmid pAzSall-tet (Fig.8) into the cleaved and trimmed SalI site of pPV1. The boxed part shows the relevant sequence of inserted cassette in detail. The resulting construct pPV12-tet is related to plasmid pPV12 (Figs. 5 and 6). A (-) directed antisense ribozyme can be obtained by transcription with T7 RNA polymerase. After excision of the tet gene with XhoI and subsequent re-ligation, the plasmid pPV12a was obtained.

Fig.11

Autoradiograph of the gelelectrophoretic analysis for cleavage of PPV target RNA by two types of antizyme RNA.
The analysis was done as described for Fig.6. Three types of RNAs were synthesized by T7 transcription : from plasmid pPV2 the PPV (-) RNA substrate (S), from plasmid pPV12 the antizyme RNA (Az) and from plasmid pPV12-tet the antizyme RNA which contains the tetracycline resistance gene inserted into the XhoI recognition sequence (Az-t). The lanes 1-3 contain the RNAs of plasmids pPV12, pPV2 and pPV12-tet, incubated at 0°C. Lanes 4-6 contain the same RNAs incubated at 60°C. Lane 7 contains the mixture between the RNAs of lane 4 and 5. In lane 8 the RNAs of lane 4

and 6 are mixed. Lane 9 is identical to 8 but the antizyme RNA is not labeled.

It is shown that the RNA derived from plasmid pPV12-tet is able to cleave the PPV (-) target RNA in the same manner as the antizyme RNA derived from plasmid pPV12.

Fig.12

Generation of a SalI-specific DNA ribozyme cassette in which the tet gene is inserted into an AflII site.

A, The plasmid pAzSal1 (Fig.8) was linearized with XhoI and the protruding ends were removed by digestion with nuclease S1.

B, In plasmid pBR322, an AflII linker (pCCTTAAGG) had been introduced subsequently at the EcoRI and AvaI sites, respectively, that had been cleaved and filled-in with Klenow polymerase, thus creating plasmid pBR-af2. It should be mentioned that the EcoRI site was restored by introduction of the AflII linker. The plasmid pBR-af2 was then cleaved with AflII that releases a DNA fragment containing the tet gene. The protruding AflII ends were filled-in with Klenow polymerase and the resulting DNA fragment recovered from an agarose gel.

C, The DNA fragment obtained in B was then ligated into the plasmid prepared according to A. The resulting construct pAzSal3-tet corresponds (except the orientation of the tet gene) to plasmid pAzSal1-tet (Fig.8), and likewise a SalI-specific DNA ribozyme cassette including tet gene can be obtained from plasmid pAzSal3-tet in analogy to pAzSal1-tet. After insertion of the DNA cassette into the SalI site of a cDNA, the tet gene can be removed by digestion with AflII.

Fig.13

The hammerhead structure with a ribozyme RNA, specific for a SalI recognition sequence, that contains an AflII and a StuI recognition sequence. The ribozyme sequence containing an AflII site is derived from DNA cassettes such as from plasmid pAzSal3-tet (Fig.12). Compared to the sequence given in Fig.4, there is another G to A mutation, that creates an AflII site within the catalytic domain.

Fig. 14

Map of plasmid pPV-BS1 and the resulting ribozyme construct pPV-BS11-tet. The map at the top shows plasmid pPV-BS1 that contains a BstYI - SphI fragment of plum pox virus (PPV) corresponding to nucleotides 3461-4058, with a SalI site at nucleotide 3631, inserted into the vector pT3T7lac (Boehringer Mannheim) from which the EcoRI site had been deleted by cleaving and subsequent filling-in followed by re-ligation. By inserting the SalI-specific DNA cassette form plasmid pAzSal3-tet (Fig.12), the construct pPV-BS11-tet was obtained that delivers after cleavage with PvuII and transcription with T3 RNA polymerase a (+) directed antisense ribozyme RNA.

Fig.15

Autoradiograph of the gelelectrophoretic analysis for cleavage of the PPV target RNA by an antisense ribozyme RNA containing the tet gene inserted into an AflII site.
Labeled target RNA was obtained from plasmid pPV-BS1 that had been linearized with HindIII and transcribed with T7 RNA polymerase. Unlabeled RNA transcript, obtained by transcription with T3 RNA polymerase from plasmid pPV-BS11-tet that had been cleaved with PvuII, was incubated with the labeled target RNA as described in Fig.6 and analyzed on a denaturating polyacrylamide gel: Lane 1, labeled RNA of pPV-BS1 not incubated; Lane 2 labeled RNA of pPV-BS1 incubated at 60°C for 30 minutes with the unlabeld RNA transcript of pPV-BS11-tet in 50 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$; Lane 3 labeled RNA of pPV-BS1 incubated at 60°C for 30 minutes in 50 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$. As demonstrated by the gel, the sequence modification into an AflII site did not influence the catalytic activity.

The following examples illustrate the invention.
More information on the methods applied herein can be found in Sambrook et al., "Molecular Cloning", Cold Spring Harbor, second edition, 1989.

Example 1

Description for the generation of antizyme RNA directed against the (-) RNA of plum pox virus (PPV), utilizing the SalI site of the cloned cDNA of PPV

The restriction enzyme SalI recognizes the palindromic sequence --GTCGAC-- . It contains within its recognition sequence a "cleavage" motif (cleavable motif or target motif) of the hammerhead RNA: GUC (= GTC, respectively). A ribozyme RNA directed against an RNA, which contains such a SalI recognition sequence, forms the hammerhead conformation as indicated in Fig.1. The objective is to create a DNA construct, which allows the transcription of such a SalI-directed ribozyme RNA. Fig.2 gives, in general form, the sequences of such a cDNA construct in comparison to the cDNA of the target. The direction of transcription is different and the boxed C/G pair within the SalI recognition sequence of the target is replaced by the catalytic domain of the ribozyme RNA. Fig.3 describes a strategy to convert the cDNA of the target into the desired ribozyme construct.

In order to test this general strategy in a practical experiment, two DNA oligonucleotides were synthesized with an automated DNA synthesizer (Applied Biosystems). The oligonucleotides comprising 25 nucleotides each had the following sequence :

Rz-Sal1 5' T T T C G G C C T C A A G G C C T C A T C A G G A 3'
Rz-Sal2 5' T C C T G A T G A G G C C T T G A G G C C G A A A 3'.

After purification of the two synthetic DNA oligonucleotides by gel-electrophoresis followed by electroelution through isotachophoresis, the DNAs were phosphorylated at the 5'-terminus with the aid of polynucleotide kinase. After this enzymatic reaction, equimolar amounts of the two labeled DNA oligonucleotides were mixed, incubated for one minute in a boiling

water bath and slowly cooled down to room temperature. Upon this de- and re-naturing step the desired double-stranded DNA cassette is formed:

```
Rz-SalI                    ---StuI ---
     5' T T T C G G C C T C A A G G C C T C A T C A G G A 3'
     3' A A A G C C G G A G T T C C G G A G T A G T C C T 5'
                           ----------                    Rz-Sal2
```

These two annealed oligodeoxynucleotides represent such a DNA cassette which is needed to create - according to the strategy outlined in Fig. 3 - a ribozyme RNA as depicted in Fig. 4, which is characterized by the presence of the StuI recognition site AGGCCT within the ribozyme sequence.

The DNA cassette consisting of the two synthetic DNA oligonucleotides Rz-Sal1 and Rz-Sal2 was used to create an antizyme RNA against the (-) RNA of plum pox virus (PPV) which is a plant virus of the potyvirus group. For this purpose, an EcoRI fragment containing 423 bases of the cDNA of (PPV), ranging from nucleotides 3409-3831 of the viral RNA genome (according to the sequence of Techeney at al., Nucleic Acids Res. 17 (1989) 10115), was cloned into the plasmid pT3T7-Sal, a modified version of the plasmid pT3T7lac (Boehringer Mannheim) from which the SalI site had been removed before, by cleaving with SalI, trimming the protruding ends with the aid of mung bean nuclease followed by re-ligation of the plasmid DNA. The EcoRI fragment was inserted in both possible orientations into this vector, so that the two plasmids pPV1 and pPV2 as given in Fig. 5 (top), were obtained. The PPV cDNA insert contains a SalI site at nucleotide 3631, i.e. 223 and 200 nucleotides apart from the EcoRI sites. The SalI site of each of the two plasmids was digested and trimmed by treatment with mung bean nuclease using the protocol as described by the supplier (N.E.Biolabs), which removes the protruding ends as outlined in Fig.3. Then, the above mentioned SalI-specific synthetic ribozyme DNA cassette, containing a StuI site, was inserted by blunt-end ligation.
The restriction enzyme StuI was used to screen the transformants for the insertion of the DNA cassette. Those plasmids which could be linearized by StuI digestion, were digested with EcoRI and the resulting DNA fragments were separated on a 5% polyacrylamide gel. By comparison with the

423 base fragment of the plasmid pPV2, recombinant plasmids could be identified which contained one, two or three copies of the SalI-specific DNA cassette inserted into the trimmed SalI site.
Those plasmids which contained more than one inserted cassette were digested with StuI and religated. Since neither the substrate cDNA nor the vector contains a StuI site, this procedure removes the cassette present in excess. However, only about 50% of the resulting plasmids can be expected to contain a correct monomeric DNA cassette, whereas in the other 50%, there are head-to-head or tail-to-tail connected parts of the DNA cassette which are useless for the generation of ribozyme RNA.

In order to screen for the orientation of insertion of the SalI-specific DNA cassette, within the two plasmids pPV1 and pPV2, a functional ribozyme assay was carried out. For this purpose, the plasmids pPV1 and pPV2 were linearized with HindIII, which cleaves that part of the polylinker next to the T3 promoter (see Fig. 5, top). Upon transcription with T7 RNA polymerase radioactively labeled PPV (+) and (-) RNA was synthesized from these two templates.
The recombinant plasmids containing the monomeric SalI-specific DNA cassette inserted and those plasmids which were obtained by StuI digestion and re-ligation, in order to eliminate excess cassettes, were digested with HindIII or PvuII, respectively. The HindIII cut DNA was transcribed with T7 RNA polymerase and the PvuII cut DNA served as template for T3 RNA polymerase. The resulting RNA transcripts were tested for ribozyme activity.
As can be deduced from Fig.3, the orientation of insertion of the SalI-specific ribozyme cassette determines whether a sense- or an antisense-directed construct will be obtained. Therefore, plasmids derived by inserting the SalI-specific ribozyme cassette into plasmid pPV1 were screened in the following way:

The RNA transcript synthesized with T3 RNA polymerase, which represents a potential sense-directed antizyme (comp. Fig.3), was incubated with PPV (+) RNA, derived from plasmid pPV1 by transcription with T7 RNA polymerase for 30 minutes at 60°C in 20 mM $MgCl_2$, 50 mM Tris pH 8.0, followed by ethanol precipitation and separation of the products on a denaturing 5% polyacrylamide gel (0,125 % bisacrylamide), containing 8M urea. Like-

wise, the RNA transcripts synthesized with T7 RNA polymerase from the same recombinant plasmids, which represent a potential antisense-directed antizyme (comp. Fig.3), were incubated with PPV (-) RNA derived from plasmid pPV2 by transcription with T7 RNA polymerase.

By using this functional assay for catalytic activity of the RNA transcripts, the recombinant clones pPV11 and pPV12 (Fig. 5, middle) were identified amongst the pPV1 derived plasmids.

Similarly, the pPV2-derived plasmids pPV21 and pPV22 (Fig. 5, bottom) could be identified, by incubating transcripts synthesized by T3 RNA polymerase with the T7 RNA transcript of plasmid pPV2 which is a PPV (-) RNA and testing the T7 RNA transcripts with the T7 RNA transcript of plasmid pPV1, respectively.
An example of a ribozyme reaction is given in Fig. 6.

Example 2

Usage of selectable markers for the construction of antizyme RNAs

For further improvement of the technique to generate antizyme constructs, an additional nucleotide exchange in the ribozyme cassette was made to create an XhoI recognition sequence which is located directly in the center of the hairpin loop of the hammerhead structure (Fig.7).
Instead of using a synthetic SalI-specific DNA cassette with a further modified sequence, the DNA oligonucleotide Az-SalI was synthesized which contained beside the sequence of the actual DNA cassette, two flanking EarI recognition sites as indicated in the top of Fig.8. In addition, the DNA oligonucleotide contained the such nucleotides at the 5'- and 3'-terminus, that it could be cloned into the plasmid pT3T7lac which had been cleaved with an enzyme that produced 5'-protruding ends (BamHI) and another enzyme that produced 3'-protruding ends (KpnI).

The sequence of the DNA oligonucleotide Az-SalI was as follows (the restriction enzyme recognition sequences contained in this oligonucleotide are indicated):

```
    BamHI              StuI              EarI
    -----              ------            ------
5' GATCCTCTTCATCCTGATGAGGCCTCGAGGCCGAAACGAAGAGGTAC 3'.
      ------             ------            -----
       EarI               XhoI              KpnI
```

The oligonucleotide was annealed to these protruding BamHI- and KpnI-ends of the vector and the ligation was done in presence of Klenow polymerase and dNTP, so that the DNA strand complementary to Az-SalI was synthesized enzymatically during the ligation reaction. Thus, the plasmid pAzSalI was created (Fig. 8, top) .
EarI is a class-IIS restriction enzyme that has its cleavage site outside of its recognition sequence, and it cleaves a DNA sequence in the following manner:

```
5' CTCTTCNNNN- 3' ->     5' CTCTTCN    NNN-  3'
3' GAGAAGNNNN- 5' ->     3' GAGAAGNNNN    -  5'.
```

Consequently, the desired SalI-specific ribozyme cassette can be obtained from plasmid pAzSalI, by digestion with EarI and subsequent filling-in the protruding ends with Klenow enzyme.

This procedure to obtain the SalI-specific DNA cassette would, as such, have no significant advantage as compared to the synthetic DNA cassette used in Example 1. But since the DNA cassette was now cloned on the plasmid pAzSalI, it was possible to take advantage of the XhoI site within the ribozyme sequence in order to introduce a marker gene.
For this purpose, an XhoI linker was introduced into the EcoRI site and also into the AvaI site of the plasmid pBR322 (after cleaving and filling-in the protruding ends, respectively), so that the tetracycline resistance gene (tet gene) could be excised by XhoI cleavage.
The resulting XhoI fragment containing the tetracycline resistance gene of pBR322, including its promoter, was introduced into the XhoI site of plasmid pAz-SalI creating the plasmid pAz-SalI-tet (a schematic map of plasmid pAz-SalI-tet is given in Fig. 9, lower part).
In order to create an antizyme RNA, the plasmid pAz-SalI-tet was digested

with EarI, the protruding ends were filled-in by Klenow polymerase and the resulting DNA fragments were separated on an agarose gel, and the DNA fragment which was the desired SalI-specific ribozyme cassette was purified by electroelution via isotachophoresis. This fragment is about 1450 bp in size and was used for ligation into plasmid pPV1, which had been cleaved with SalI and treated with mung bean nuclease in order to remove the protruding ends as described under Example 1.

Ampicillin and tetracycline were used to select for transformants.

The recombinant plasmids were analyzed for the presence of the StuI site, which is characteristic for the SalI-specific ribozyme cassette, and for the presence of the XhoI fragment containing the tetracycline resistance gene. By digesting the recombinant plasmids with restriction enzymes that cleave within the tetracycline resistance gene and also in the sequences derived from plasmid pPV1, e.g. HindIII or BamHI, is was possible to deduce the orientation in which the SalI-specific DNA cassette had been inserted. In this way, the plasmids pPV11-tet and pPV12-tet were identified, which are related to plasmids pPV11 and pPV12 (Fig. 5, middle), but contain, in addition, the tetracycline resistance gene within the ribozyme sequence.

Before excising the tetracycline resistance gene from the plasmids, by XhoI digestion, plasmid pPV12-tet (Fig.10) was tested whether it already could deliver a functional antizyme RNA such as the RNA given at the bottom of Fig. 9. Therefore, the plasmid was cleaved with XbaI and transcribed with T7 RNA polymerase and the resulting RNA was incubated with the target RNA derived from plasmid pPV2 by T7 transcription. Surprisingly, the insertion of the tetracycline resistance gene into the ribozyme cassette did not affect the catalytic activity of the ribozyme (Fig. 11).

The experiment shows that the catalytic domain of the ribozyme which does not form base pairs with the target RNA (sequence (a)), which consists of 22 nucleotides in case of plasmid pPV12, can be divided into two sub-domains of 9 and 13 nucleotides, respectively, and separated by more than 1400 bases in pPV12-tet, without loss of catalytic activity.

After cleavage of the plasmid pPV12-tet with XhoI and re-ligation, the tetracycline resistance gene was removed and plasmid pPV12a was obtained. This plasmid is very similar to plasmid pPV12. The only difference is that it contains an XhoI site according to Fig. 7, whereas plasmid pPV12

contains a ribozyme sequence according to Fig. 4.

The addition of the tetracycline resistance gene into the portable ribozyme cassette has two major advantages :

(i) It drastically reduces screening work, in order to identify antizyme clones, because it can be selected for tetracycline resistance.

(ii) It can be discriminated between sense or antisense directed antizyme constructs, according to the orientation of the tetracycline resistance gene by simple restriction analysis.

Once the antizyme construct has been made, the tetracycline resistance gene can be removed by digestion with XhoI, but the antizyme RNA also works in the presence of it.

Example 3

Construction of the plasmids pAzBam1, pAzBam1-tet, pAzCla1, pAzCla1-tet, pAzEco1, pAzEco1-tet, pAzKpn1, pAzKpn1-tet, pAzXba1, pAzba1-tet, pAzXba2 and pAzXba2-tet from which DNA ribozyme cassettes with and without tet gene can be prepared that are suitable for the insertion into different restriction sites and the application of these cassettes to generate further ribozymes

To extend the spectrum of available DNA cassettes that can be utilized to generate antisense ribozymes by incorporation into cDNA, several DNA oligodeoxynucleotides, similar as AzSal1 given in Example 2, were cloned in order to create constructs which correspond to pAzSal1 and pAzSal1-tet. The following list shows the relevant sequence of the resulting plasmids that contain DNA cassettes that are located between two EarI sites, so tha they can be released by digestion with that class-IIs restriction enzyme, followed by filling-in the protruding ends. Each construct exists with and without tet gene inserted into the XhoI site (that is marked below), so that DNA cassettes with and without tet gene can be obtained. The sequence of the "core ribozyme" corresponds to sequence (a):

pAzSal1, pAzSal1-tet

```
                5'<- SalI specific nuc. -> 3'
    KpnI         !      core ribozyme      !!         BamHI
   ------        ! ---------------------- !!        ------
5' GGTACCTCTTCG T TTCGGCCTCGAGGCCTCATCAG GA TGAAGAGGATCC 3'
3' CCATGGAGAAGC A AAGCCGGAGCTCCGGAGTAGTC CT ACTTCTCCTAGG 5'
        ------           ------                ------
         EarI             XhoI                  EarI
```

pAzBam1, pAzBam1-tet

```
                  5'<- BamHI specific nuc.
    KpnI         !!!    core ribozyme              BamHI
   ------        !!! ----------------------       ------
5' GGTACCTCTTCG GAT TTCGGCCTCGAGGCCTCATCAG TGAAGAGGATCC 3'
3' CCATGGAGAAGC CTA AAGCCGGAGCTCCGGAGTAGTC ACTTCTCCTAGG 5'
        ------            ------             ------
         EarI              XhoI               EarI
```

pAzCla1, pAzCla1-tet

```
                ClaI specific nuc. -> 3'
    KpnI           core ribozyme                BamHI
   ------        ---------------------- !       ------
5' GGTACCTCTTCG TTCGGCCTCGAGGCCTCATCAG G TGAAGAGGATCC 3'
3' CCATGGAGAAGC AAGCCGGAGCTCCGGAGTAGTC C ACTTCTCCTAGG 5'
        ------        ------               ------
         EarI          XhoI                 EarI
```

pAzEco1, pAzEco1-tet

```
                  5'<- EcoRI specific nuc.
    KpnI         !!!    core ribozyme              BamHI
   ------        !!! ----------------------       ------
5' GGTACCTCTTCG AAT TTCGGCCTCGAGGCCTCATCAG TGAAGAGGATCC 3'
3' CCATGGAGAAGC TTA AAGCCGGAGCTCCGGAGTAGTC ACTTCTCCTAGG 5'
        ------            ------             ------
         EarI              XhoI               EarI
```

pAzKpnl, pAzKpnl-tet

```
                5'<- KpnI specific nuc. -> 3'
    KpnI         !!    core ribozyme       !        BamHI
   ------        !! ---------------------- !       ------
5' GGTACCTCTTCG GT TTCGGCCTCGAGGCCTCATCAG C TGAAGAGGATCC 3'
3' CCATGGAGAAGC CA AAGCCGGAGCTCCGGAGTAGTC G ACTTCTCCTAGG 5'
       ------           ------              ------
        EarI             XhoI                EarI
```

pAzXbal, pAzXbal-tet

XbaI: NTC type

```
                XbaI specific nuc. -> 3'
    KpnI           core ribozyme                BamHI
   ------        ---------------------- !!!      ------
5' GGTACCTCTTCG TTCGGCCTCGAGGCCTCATCAG TAG TGAAGAGGATCC 3'
3' CCATGGAGAAGC AAGCCGGAGCTCCGGAGTAGTC ATC ACTTCTCCTAGG 5'
       ------         ------                ------
        EarI           XhoI                  EarI
```

pAzXba2, pAzXba2-tet

XbaI CTA type

```
              5'<-- XbaI specific nuc. -> 3'
   KpnI        !!    core ribozyme       !        BamHI
  ------       !! ---------------------- !       ------
5' GGTACCTCTTCG CT TTCGGCCTCGAGGCCTCATCAG G TGAAGAGGATCC 3'
3' CCATGGAGAAGC GA AAGCCGGAGCTCCGGAGTAGTC C ACTTCTCCTAGG 5'
       ------           ------              ------
        EarI             XhoI                EarI
```

With the aid of these constructs, it was possible to generate ribozymes that were directed against the mRNA of the ice nucleation gene (inaZ) and hrpS gene of Pseudomonas syringae (SalI sites), the white gene of Drosophila melanogaster (SalI site), the transcriptional regulator GCN4 of yeast (BstEII site, in that case a synthetic cassette without tet gene was used). Ribozymes were also created against the (+) and (-) RNA of Sendai virus by inserting DNA cassettes into two different BstBI sites,

and both possible cassettes for the XbaI site (L gene of the virus) and a SalI site (P gene).
In all cases, catalytic antisense ribozymes could be obtained after the incorporation of the corresponding DNA cassettes.

Example 4

Construction of the plasmids pAzBam3-tet, pAzCla3-tet, pAzSal3-tet, pAzKpn3-tet, pAzXba3-tet, pAzXba4-tet from which DNA ribozyme cassettes can be excised that contain the tet gene inserted into an AflII site within the sequence of the catalytic domain

A potential complication of the technique to incorporate selectable DNA cassettes with a selectable marker gene arises when the cDNA itself contains an XhoI site. Then, a DNA cassette, such as the one derived from plasmid pAzSall-tet, can still be incorporated into any site of interest. However, the excision of the tet gene, that is cloned into the XhoI site within the catalytic domain (compare Fig.7), requires to prepare partially digested DNA. The same complication arises when a second cassette is to be introduced into a cDNA construct that already contains a DNA cassette with a ribozyme domain including an XhoI site. In order to overcome this inconvenience, a second series of recombinant plasmids was prepared that contained the tet gene within an AflII site. For this purpose, the plasmid pAzSall (compare Fig.8, top) was digested with XhoI, and the resulting protruding ends were removed by trimming with nuclease S1 (Fig.12A). In parallel, a synthetic AflII linker (CCTTAAGG) was consecutively introduced into the EcoRI and AvaI sites of the plasmid pBR322 after filling-in the corresponding protruding ends, respectively. From this modified plasmid pBR-af2 (Fig.12B), an AflII fragment, containing the tet gene, was released and the protruding ends filled-in with Klenow polymerase. After purification on an agarose gel and subsequent electroelution, the DNA fragment was inserted into the XhoI-cleaved and trimmed plasmid pAzSall (Fig.12C). Thus, the AflII site C'TTAAG was restored. As a result of this manipulation, the XhoI site within the catalytic domain is replaced by the AflII site. Therefore, a second type of DNA cassettes could be obtained which can be introduced into cDNAs that contain an XhoI site or already an inserted ribozyme cassette with an XhoI site. After

excision of the AflII fragment containing the tet gene, a ribozyme as given in Fig.13 is obtained.

The following plasmids, each containing a tet gene inserted into the AflII site, were generated :

pAzSal3-tet

```
                5'<- SalI specific nuc. -> 3'
    KpnI         !     core ribozyme      !!        BamHI
   ------        ! ---------------------- !!       ------
5' GGTACCTCTTCG T TTCGGCCTTAAGGCCTCATCAG GA TGAAGAGGATCC 3'
3' CCATGGAGAAGC A AAGCCGGAATTCCGGAGTAGTC CT ACTTCTCCTAGG 5'
        ------          ------               ------
         EarI            AflII               EarI
```

pAzBam3-tet

```
                  5'<- BamHI specific nuc.
    KpnI         !!!    core ribozyme             BamHI
   ------        !!! ----------------------       ------
5' GGTACCTCTTCG GAT TTCGGCCTTAAGGCCTCATCAG TGAAGAGGATCC 3'
3' CCATGGAGAAGC CTA AAGCCGGAATTCCGGAGTAGTC ACTTCTCCTAGG 5'
        ------          ------              ------
         EarI            AflII              EarI
```

pAzCla3-tet

```
                ClaI specific nuc. -> 3'
    KpnI           core ribozyme               BamHI
   ------        ---------------------- !       ------
5' GGTACCTCTTCG TTCGGCCTTAAGGCCTCATCAG G TGAAGAGGATCC 3'
3' CCATGGAGAAGC AAGCCGGAATTCCGGAGTAGTC C ACTTCTCCTAGG 5'
        ------          ------              ------
         EarI            AflII              EarI
```

pAzKpn3-tet

```
                5'<- KpnI specific nuc. -> 3'
    KpnI         !!    core ribozyme       !        BamHI
   ------        !! ---------------------- !       ------
5' GGTACCTCTTCG GT TTCGGCCTTAAGGCCTCATCAG C TGAAGAGGATCC 3'
3' CCATGGAGAAGC CA AAGCCGGAATTCCGGAGTAGTC G ACTTCTCCTAGG 5'
        ------           ------              ------
         EarI             AflII               EarI
```

pAzXba3-tet

XbaI: NTC type

```
                XbaI specific nuc. -> 3'
    KpnI           core ribozyme                BamHI
   ------        ---------------------- !!!      ------
5' GGTACCTCTTCG TTCGGCCTTAAGGCCTCATCAG TAG TGAAGAGGATCC 3'
3' CCATGGAGAAGC AAGCCGGAATTCCGGAGTAGTC ATC ACTTCTCCTAGG 5'
        ------        ------                  ------
         EarI          AflII                   EarI
```

pAzXba4-tet

XbaI CTA type

```
               5'<- XbaI specific nuc. -> 3'
    KpnI         !!    core ribozyme       !        BamHI
   ------        !! ---------------------- !       ------
5' GGTACCTCTTCG CT TTCGGCCTTAAGGCCTCATCAG G TGAAGAGGATCC 3'
3' CCATGGAGAAGC GA AAGCCGGAATTCCGGAGTAGTC C ACTTCTCCTAGG 5'
        ------           ------              ------
         EarI             AflII               EarI
```

Example 5

A ribozyme containing an AflII recognition sequence in its catalytic domain

From the plasmid pAzSal3-tet, a SalI-specific DNA cassette was prepared by digestion with Ksp632I (isoschizomer of EarI) and filling-in the resulting protruding ends. This SalI-specific DNA cassette was purified on an agarose gel and electroeluted. In parallel, one μg of plasmid pPV-BS1 (Fig.14), which contains a fragment of the cDNA of plum pox virus (PPV) was cleaved with SalI. The plasmid was dissolved in 20 μl of S1 buffer (225 mM NaCl, 30 mM potassium acetate pH 4.5, 200 μM $ZnSO_4$ and 5% glycerol) and treated with 2 units of nuclease S1 (Boehringer Mannheim) for 20 minutes on ice. Then 5 μl of S1 Stop buffer (300 mM Tris/HCl, 50 mM EDTA, pH 8.0) were added and the sample incubated for 10 minutes at 65 °C, followed by phenolization and precipitation with isopropanol. Compared to the construction of the ribozymes described in Examples 1 and 2, the above process represents a preferred embodiment with respect to reaction conditions for the trimming reaction. About 300 ng of the plasmid pPV-BS1, treated in that way, was ligated with about 100 ng of the above mentioned SalI-specific DNA cassette prepared from plasmid pAzSal3-tet in a volume of 5 μl of blunt end ligation buffer (50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 5% PEG 8000, 1 mM DTT and 100 μM ATP containing 3 units of T4 DNA ligase (Minotech, Heraklion, Greece) for 16 hours at 12°C. After transformation in E.coli strain JM83, and selection for ampicillin and tetracycline, the resulting clones were analyzed by restriction mapping. Thus the plasmids pPV-BS11-tet (Fig. 14) and pPV-BS12-tet were identified that deliver a (+) or (-) directed ribozyme, respectively. Fig. 15 shows an autoradiograph, confirming the catalytic activity of the transcript obtained from pPV-BS11-tet.

Example 6

Stabilisation of the antisense ribozyme RNA by insertion of a DNA cassette that forms after transcription a stem-loop structure

In order to protect the ribozyme after expression in vivo, two synthetic DNA oligonucleotides were synthesized:

Stem1: 5' GCGGCCGCTCGGGCCACGCGAGGCCCGTGCGGCCGT 3'
and
Stem2: 5' ACGGCCGCACGGGCCTCGCGTGGCCCGAGCGGCCGC

which after phosphorylation with T4 polynucleotidkinase and subsequent annealing can form the following double-stranded DNA cassette:

```
5' pGCGGCCGCTCGGGCCACGCGAGGCCCGTGCGGCCGT  3'
3'  CGCCGGCGAGCCCGGTGCGCTCCGGGCACGCCGGCAp 5' .
```

The DNA cassettes contains restriction sites for AvaI, EagI,

```
           AvaI            AvaI
          ------          ------
5' pGCGGCCGCTCGGGCCACGCGAGGCCCGTGCGGCCGT  3'
3'  CGCCGGCGAGCCCGGTGCGCTCCGGGCACGCCGGCAp 5' .
    ------                        ------
     EagI                          EagI
```

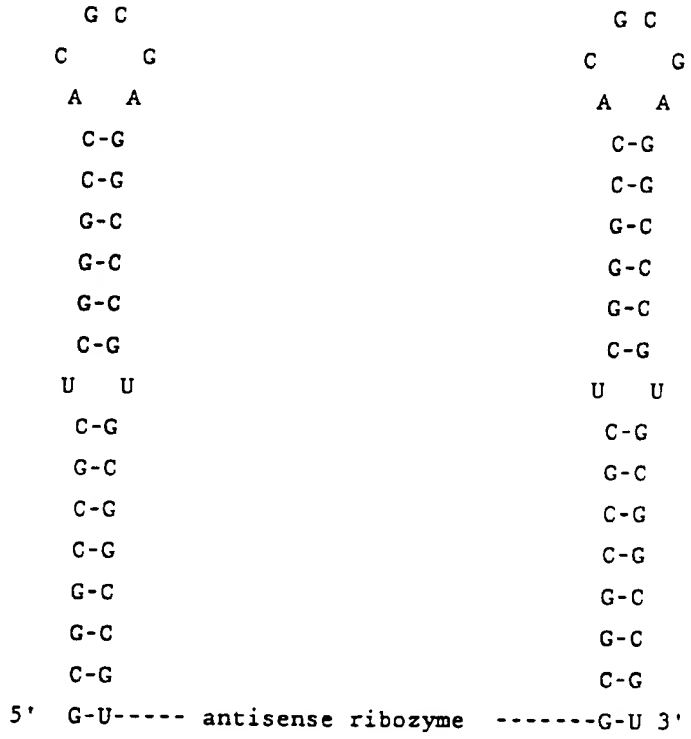
and for the restriction endonucleases NotI, SfiI that recognize a sequence of 8 bases:

```
    NotI
   --------
5' pGCGGCCGCTCGGGCCACGCGAGGCCCGTGCGGCCGT  3'
3'  CGCCGGCGAGCCCGGTGCGCTCCGGGCACGCCGGCAp 5' .
     ----.....----      ----.....----
          SfiI               SfiI
```

The DNA cassette can be inserted by blunt-end ligation into the DNA con-

struct that encodes an antisense ribozyme at two sites, up- and downstream of the inserted DNA ribozyme cassette(s). The restriction sites allow to detect the insertion of the cassette and to test for the orientation of insertion and also the excision of excess cassettes. Preferred are the same orientations of insertion at the 5'-terminus and the 3'-terminus, respectively.

The sequence of the DNA cassette was chosen to ensure that when the DNA containing the DNA cassette composed of Steml and Stem2 is used as a template for transcription, the resulting RNA will assume a stem loop structure as shown below:



The sequence of the DNA cassette was also chosen, so that the resulting RNA stem is not completely double-stranded to avoid degradation by double-strand-specific endoribonucleases of the RNaseIII-type. The stem-loop structures at the 5' and 3'- ends of the antisense ribozyme RNA provide protection against exonucleases. Similar structures, that are highly stable in vivo, are found amongst bacterial IS elements; see Simons and Kleckner, Annu. Rev Genet. 22 (1988), 567-700.

The RNA transcript can assume an alternative structure involving the 5'- and 3'-terminal regions derived form the cloned Stem cassette by forming a "folding back" structure (ping pong paddle). Thus a "pseudo-circular" structure can be formed in which the 5'-end and the 3'-end of the antisense ribozyme basepair, without forming a completely double-stranded RNA segment. Also this structure confers protection against exonuclease attack:

```
            U     A   A     U
5'  CGCGGCCGC CGGGCC CGCG GGCCCG GCGGCCGU-----antisense -------
    ::::::::: :::::: :::: :::::: ::::::::                    :
3'  GCGCCGGCG GCCCGG GCGC CCGGGC CGCCGGCA------ribozyme--------
            U     A   A     U
```

The plasmids pPV-BS1 and pPV1 were deposited in E. coli JM83 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, under the requirements of the Budapest Treaty under the deposition numbers DSM 6624 and DSM 6625, respectively, on July 26, 1991.

Claims

1. A portable ribozyme cassette displaying the following features:

   (a) a DNA sequence (a) encoding that part of a ribozyme which does not form base pairs with the target RNA; and

   (b) DNA sequences (b) flanking the DNA sequence (a), which are derived from the protruding ends of a restriction enzyme cleavage site of a DNA sequence encoding a target RNA, and which encode a part of that part of a ribozyme which forms base pairs with the target RNA,

   wherein after insertion of the ribozyme cassette into a target DNA that encodes the target RNA, said DNA sequences (a) and (b) encode together with that strand of said target DNA that is complementary to the target RNA (antisense strand), a ribozyme, having endoribonuclease activity for said target RNA.

2. The portable ribozyme cassette according to claim 1, wherein said DNA sequences (a) and (b) are represented by the following general formula:

$$\begin{array}{ll} 5' & M_2\ M_1\ K\ L\ T\ T\ C\ G^1(X)_x\ C^2Y_y\ T\ C\ W\ T\ C\ A\ G\ N_1\ N_2\ N_3\ N_4 \quad 3' \\ 3' & M'_2M'_1K'L'A\ A\ G\ C^1(X')_xG^2Y_y'A\ G\ W'A\ G\ T\ C\ N'_1N'_2N'_3N'_4 \quad 5' \end{array}$$

in which said DNA sequence (a) is represented by the sequence:

$$\begin{array}{ll} 5' & T\ T\ C\ G^1(X)_x\ C^2Y_y\ T\ C\ W\ T\ C\ A\ G \quad 3' \\ 3' & A\ A\ G\ C^1(X')_xG^2Y_y'A\ G\ W'A\ G\ T\ C \quad 5' \end{array}$$

and said DNA sequence (b) is represented by the sequences:

$$\begin{array}{lll} 5'\ M_2\ M_1\ K\ L & \text{and} & N_1\ N_2\ N_3\ N_4\ 3' \\ 3'\ M'_2M'_1K'L' & & N'_1N'_2N'_3N'_4\ 5' \end{array}$$

wherein:

the nucleotides K and L represent the first and second nucleotide (5'-3') of the target motif of the target RNA, and the nucleotides M_1 and N_1 are the first nucleotides flanking the target motif of the target RNA at the 5'- and 3'- side, respectively;
the nucleotides M_2 and N_2 are the second nucleotides flanking the target motif of the target RNA at the 5'- and 3'- side, respectively, and the nucleotides N_3 and N_4 are located in the third and fourth position at the 3'-side of the target motif of the target RNA;
the nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4, independently, are A,G,C or T or not present in the cassette, under the proviso that
the presence of M_2 requires the presence of M_1,
the presence of M_1 requires the presence of K,
the presence of K requires the presence of L,
the presence of N_4 requires the presence of N_3,
the presence of N_3 requires the presence of N_2,
the presence of N_2 requires the presence of N_1,
and under the proviso that the total number of said nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4 is 0 to 4, preferably 1 to 4;
W, X or Y is A, G, C or T;
x is at least 6, wherein each X independently is A, G, C or T and wherein X is selected so that the complementary nucleotides X' form at least one additional base pair next to the base pair formed by the nucleotides C^1 and G^2 (which flank the sequence (X')) in the secondary structure which is formed when the ribozyme pairs to its target sequence;
y is 0 or 1;
when the nucleotide C^1 is C, the nucleotide G^1 is G, when the nucleotide C^1 is T, the nucleotide G^1 is A, when nucleotide C^1 is C or T, the nucleotides C^2 and G^2 are either C and G or T and A, respectively;
the nucleotides marked with " ' " are complementary nucleotides;
and
the nucleotides M_2, M_1, K, L, N_1, N_2, N_3, and N_4 correspond to nucleotides from the protruding ends obtained after cleavage of said restriction enzyme cleavage site with the corresponding restriction enzyme, said restriction enzyme cleavage site containing in said

protruding ends an additional nucleotide Z which is A,G,C or T.

3. The portable ribozyme cassette according to claim 1 or 2, wherein L is T.

4. The portable ribozyme cassette according to any one of claims 1 to 3, wherein M_1, K, L, N_1, N_2, N_3 and N_4 have any of the following meanings:

	M_1	K	L	N_1	N_2	N_3	N_4
1	-	-	-	G	-	-	-
2	-	-	-	T	-	-	-
3	-	-	T	A	-	-	-
4	-	G	T	-	-	-	-
5	G	A	T	-	-	-	-
6	-	C	T	G	-	-	-
7	-	G	T	C	-	-	-
8	-	-	T	G	A	-	-
9	-	-	T	G	G	-	-
10	-	-	-	A	T	G	-
11	-	-	-	C	G	G	-
12	-	-	-	T	A	G	-
13	-	G	T	A	C	-	-
14	-	-	-	C	A	G	G
15	-	-	-	C	T	G	G
16	-	-	T	C	A	-	-
17	-	-	T	-	-	-	-
18	-	-	-	A	-	-	-
19	-	-	T	A	A	-	-
20	-	T	T	A	-	-	-
21	A	A	T	-	-	-	-

5. The portable ribozyme cassette according to any one of claims 1 to 4, wherein Y or W is selected such that it forms a restriction enzyme cleavage site together with the neighboring nucleotides.

6. The portable ribozyme cassette according to any one of claims 1 to 5, wherein X is selected such that it forms a restriction cleavage site either alone or together with the neighboring nucleotides.

7. The portable ribozyme cassette according to claim 6, wherein a marker sequence is contained in said restriction enzyme cleavage site.

8. The portable ribozyme cassette according to claim 7, wherein said marker sequence is a selectable marker gene.

9. A DNA sequence encoding a ribozyme (antizyme), said DNA sequence containing a portable ribozyme cassette according to any one of claims 1 to 8 in an orientation allowing the production of ribozyme displaying endoribonuclease activity upon expression in a host cell or when used as a template in a polymerase chain reaction, and containing additional sequences flanking said ribozyme cassette which have a length sufficient to provide a target specific endoribonuclease activity of the encoded ribozyme.

10. A DNA sequence according to claim 9, where in said additional sequences flanking said ribozyme cassette and having a length sufficient to provide a target specific endoribonuclease activity of the encoded ribozyme themselves contain at least one DNA domain that after transcription into RNA assumes a stable stem-loop structure.

11. The DNA sequence according to claim 10, wherein said additional DNA domain forming stem-loop structures is derived from a synthetic double-stranded DNA cassette having the sequence:

5' pGCGGCCGCTCGGGCCACGCGAGGCCCGTGCGGCCGT 3'
3' CGCCGGCGAGCCCGGTGCGCTCCGGGCACGCCGGCAp 5' .

12. A recombinant vector containing a portable ribozyme cassette according to any one of claims 1 to 8 or a DNA sequence according to any of claims 9 to 11.

13. The recombinant vector according to claim 12, wherein said portable ribozyme cassette is flanked by restriction sites allowing its precise excision from the recombinant vector.

14. The recombinant vector according to claim 12 or 13 wherein said DNA sequence is under the control of a suitable promoter.

15. A host organism containing a recombinant vector according to any one of claims 12 to 14.

16. A method for the production of a ribozyme, which comprises cultivating a host according to claim 15 under suitable conditions and isolating said ribozyme from the culture.

17. A ribozyme encoded by a DNA sequence according to any one of claims 9 to 11.

18. A viral, bacterial, plant or animal genome containing a portable ribozyme cassette according to any one of claims 1 to 8 or a DNA sequence according to any one of claims 9 to 11.

19. A virus, bacterium, fungus, plant or animal containing a genome according to claim 18.

20. A composition containing a ribozyme according to claim 17 or a DNA sequence according to any one of claims 9 to 11, optionally in association with a pharmaceutically, veterinarially or agriculturally acceptable carrier and/or excipient.

21. The composition according to claim 20 for the suppressing the undesired activity of a gene or for eliminating the disease-causing capability of an infectious agent.

22. The composition according to claim 20 or 21, wherein said DNA sequence is contained in a carrier vector, preferably in a retrovirus or in a vaccinia virus.

23. A method for the production of a DNA sequence encoding a ribozyme, said DNA sequence containing a portable ribozyme cassette according to any one of claims 1 to 8, comprising the steps of:

(A) selecting in a DNA sequence encoding a desired target RNA, which is to be inactivated by a ribozyme, a restriction enzyme cleavage site of the following nucleotide sequence:

$M_2\ M_1\ K\ L\ Z\ N_1\ N_2\ N_3\ N_4$,

wherein M_2, M_1, K, L, N_1, N_2, N_3, and N_4, have the same meaning as given in the preceding claims;
Z is A, G, C or T;
Z corresponds to the 3'-terminal nucleotide of the 5'-terminal ribozyme cleavage product of the target RNA;
the nucleotide sequence K L Z corresponds to a nucleotide sequence of the target RNA which is cleavable by a ribozyme;
and wherein Z is part of the protruding ends obtained after cleavage of said restriction enzyme cleavage site with the corresponding restriction enzyme;

(B) cleaving said restriction enzyme cleavage site of the DNA sequence given in (A) with the corresponding restriction enzyme;

(C) removing the protruding ends of the cleavage product of (B) and creating blunt ends;

(D) producing a ribozyme cassette by carrying out a method comprising the following steps :

(DA) adding to the 5'-terminus of a DNA sequence (a), as defined in the preceding claims, the nucleotides located at the 5'-side of Z in the protruding ends as obtained in (B); and

(DB) adding to the 3'-terminus of said DNA sequence (a) the nucleotides located at the 3'-side of Z in said protruding ends;

(E) insertion of the ribozyme cassette obtained in step (D) into the DNA sequence obtained in step (C).

24. The method according to claim 23, wherein the portable ribozyme cassette displaying the structure given in step (D), which is inserted in step (E) into the DNA sequence obtained in step (C), is excised from a cloning vector by cleavage of restriction enzyme cleavage sites flanking said portable ribozyme cassette.

25. A method for the production of a portable ribozyme cassette according to any one of claims 1 to 8, comprising the steps of:

(A) selecting in a DNA sequence encoding a desired target RNA, which is to be inactivated by a ribozyme, a restriction enzyme cleavage site of the following nucleotide sequence:

$M_2\ M_1\ K\ L\ Z\ N_1\ N_2\ N_3\ N_4$,

wherein M_2, M_1, K, L, N_1, N_2, N_3, and N_4, have the same meaning as given in the preceding claims;
Z is A, G, C or T;
Z corresponds to the 3'-terminal nucleotide of the 5'-terminal ribozyme cleavage product of the target RNA;
the nucleotide sequence K L Z corresponds to a nucleotide sequence of the target RNA which is cleavable by a ribozyme;
and wherein Z is part of the protruding ends obtained after cleavage of said restriction enzyme cleavage site with the corresponding restriction enzyme;

(B) determining the protruding ends of said restriction enzyme cleavage site which are created by cleaving with the corresponding restriction enzyme; and

(C) producing a ribozyme cassette by carrying out a method comprising the following steps :

(CA) adding to the 5'-terminus of a DNA sequence (a), as defined

in the preceding claims, the nucleotides located at the 5'-side of Z in the protruding ends determined in (B); and

(CB) adding to the 3'-terminus of said DNA sequence (a) the nucleotides located at the 3'-side of Z in said protruding ends.

26. A method of treating a human being, an animal or a plant in need thereof against a disease caused by the undesired activity of a gene or by an infectious agent, said method comprising administering a composition according to any one of claims 20 to 22.

Ribozyme directed against an RNA with a SalI recognition sequence

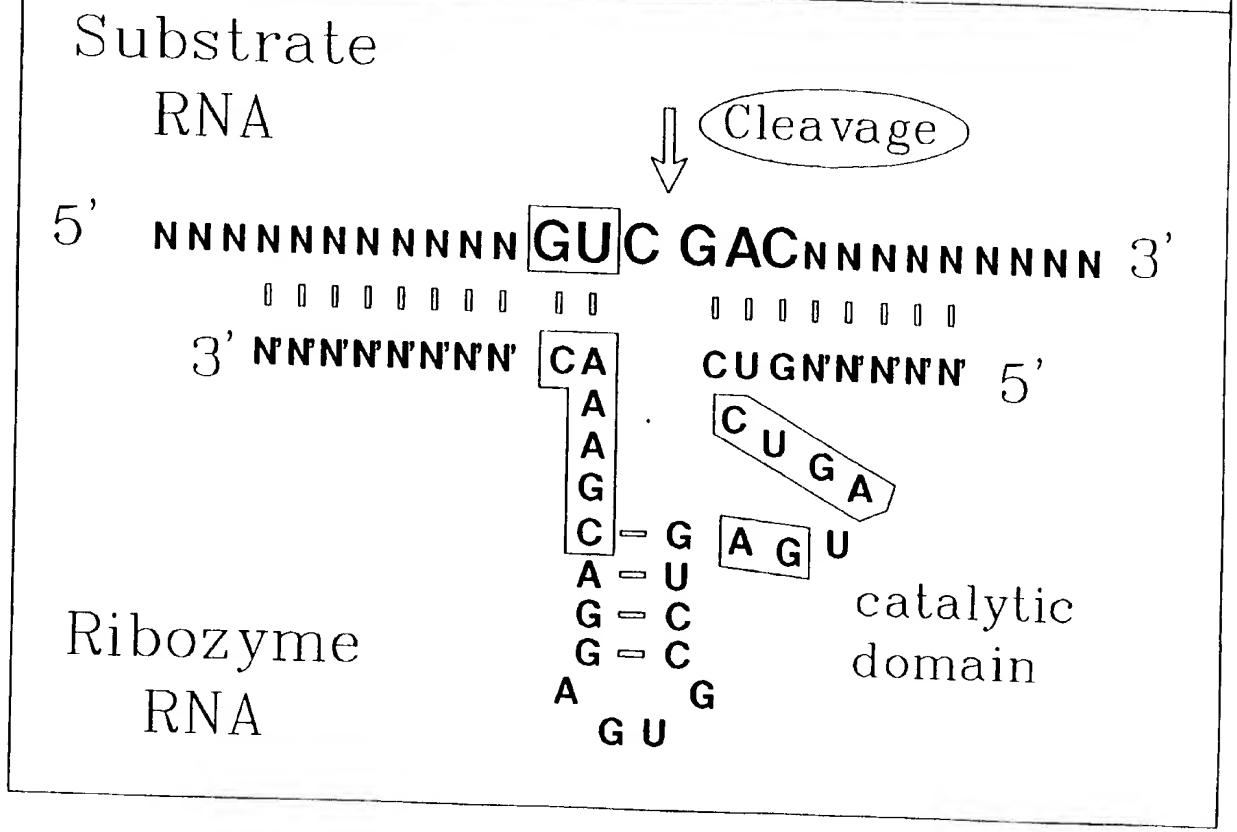


Figure 1

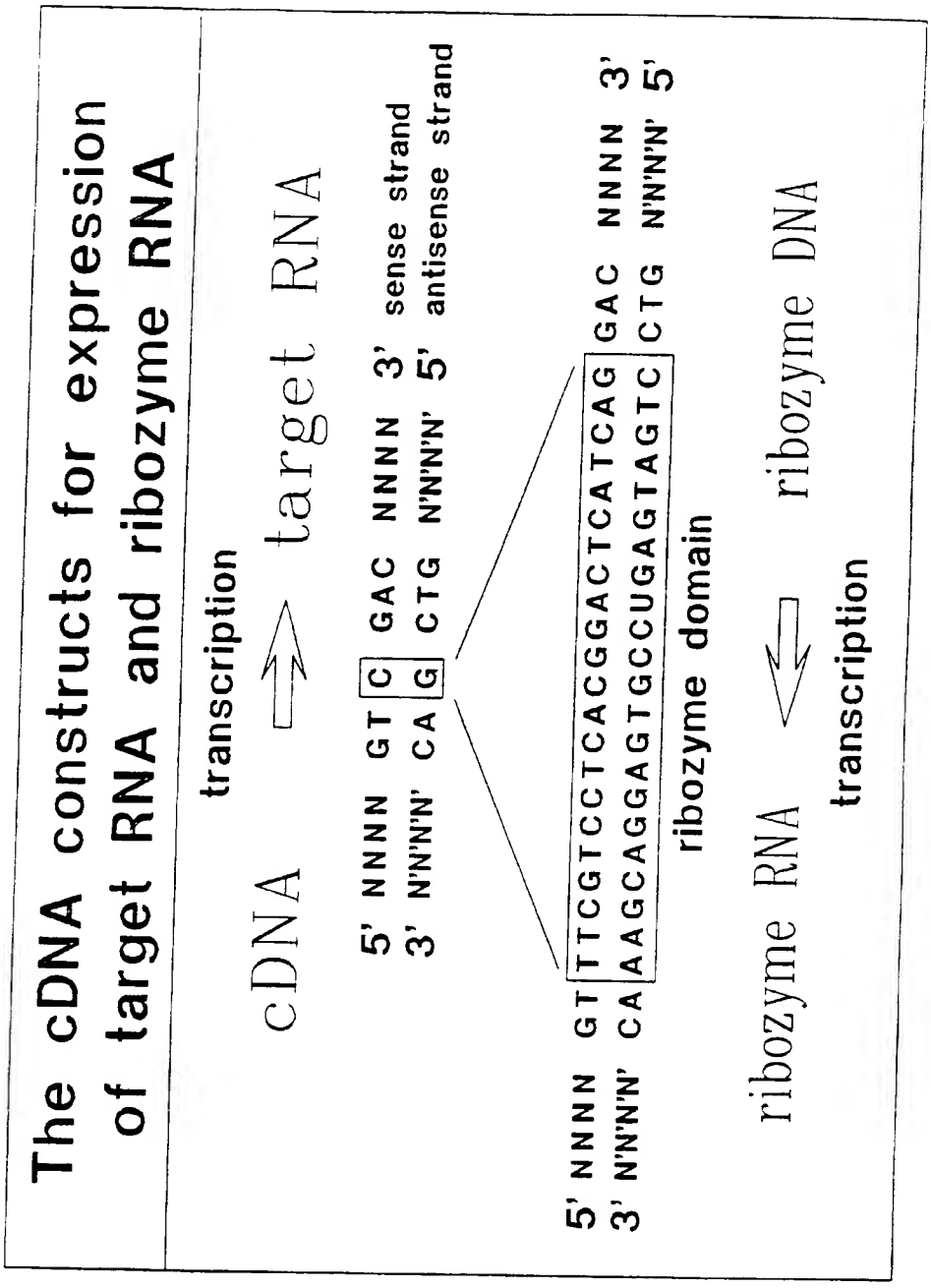

The cDNA constructs for expression of target RNA and ribozyme RNA
transcription
cDNA
target RNA
5' NNNN GT C GAC NNNN 3' sense strand
3' N'N'N'N' CA G CTG N'N'N'N' 5' antisense strand
5' NNNN GT TTCGTCCTCACGGACTCATCAG GAC NNNN 3'
3' N'N'N'N' CA AAGCAGGAGTGCCUGAGTAGTC CTG N'N'N'N' 5'
ribozyme domain
ribozyme RNA
ribozyme DNA
transcription

Figure 2

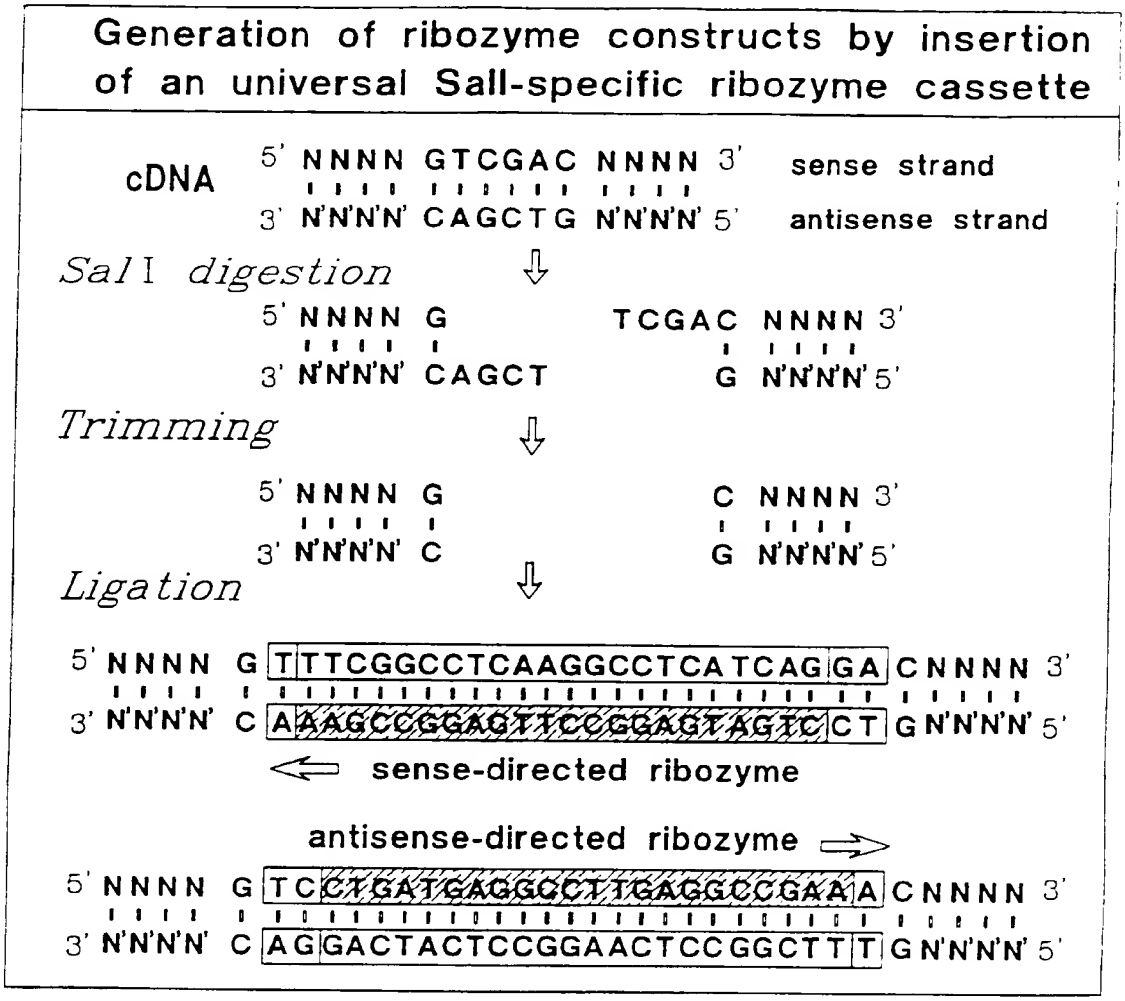

Generation of ribozyme constructs by insertion of an universal SalI-specific ribozyme cassette
cDNA
5' NNNN GTCGAC NNNN 3'
3' N'N'N'N' CAGCTG N'N'N'N' 5'
sense strand
antisense strand
SalI digestion
5' NNNN G
TCGAC NNNN 3'
3' N'N'N'N' CAGCT
G N'N'N'N' 5'
Trimming
5' NNNN G
C NNNN 3'
3' N'N'N'N' C
G N'N'N'N' 5'
Ligation
5' NNNN G TTTCGGCCTCAAGGCCTCATCAG GA CNNNN 3'
3' N'N'N'N' C AAAGCCGGAGTTCCGGAGTAGTC CT GN'N'N'N' 5'
sense-directed ribozyme
antisense-directed ribozyme
5' NNNN G TC CTGATGAGGCCTTGAGGCCGAAA CNNNN 3'
3' N'N'N'N' C AG GACTACTCCGGAACTCCGGCTT T GN'N'N'N' 5'

Figure 3

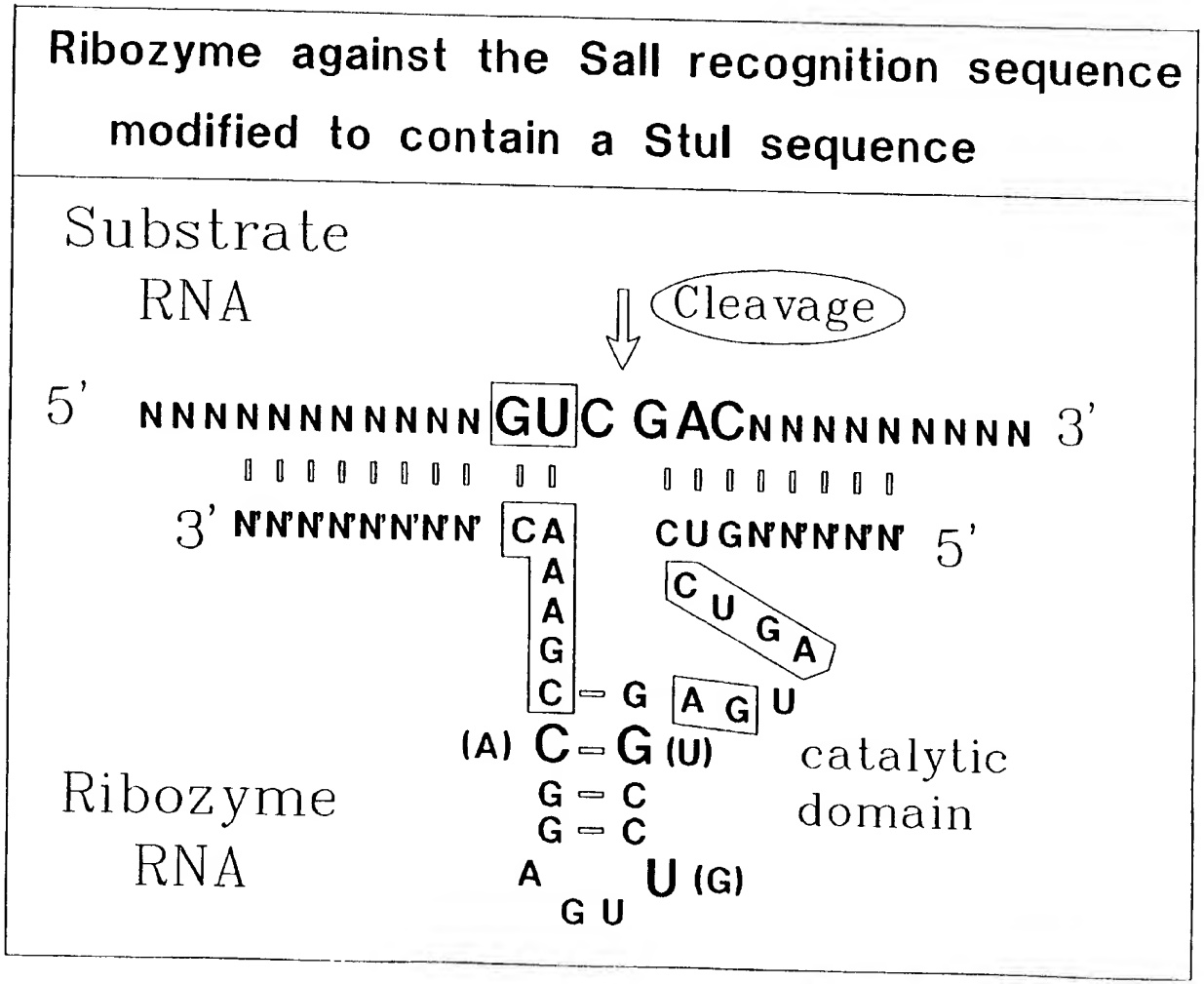
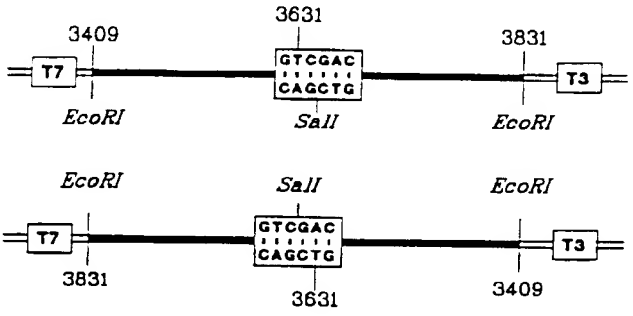

Ribozyme against the SalI recognition sequence modified to contain a StuI sequence
Substrate RNA
Cleavage
5' NNNNNNNNNNN GUC GACNNNNNNNNN 3'
3' NNNNNNNNNNN CA CUGNNNNNNN 5'
A
A
G
C
C U G A
G
A G U
(A) C G (U)
G C
G C
A U (G)
G U
Ribozyme RNA
catalytic domain

Figure 4

The plasmids pPV1 and pPV2 and the resulting antizyme constructs

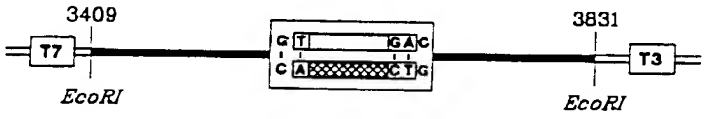


pPV1

T7 : PPV (+) RNA
T3 : PPV (-) RNA

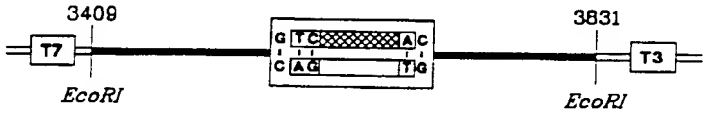
pPV2

T7 : PPV (-) RNA
T3 : PPV (+) RNA



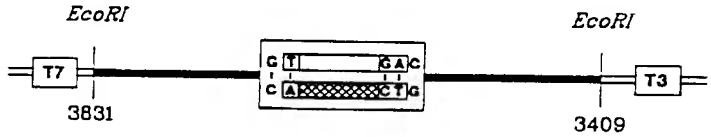
pPV11

T3 : (+) directed
PPV antizyme



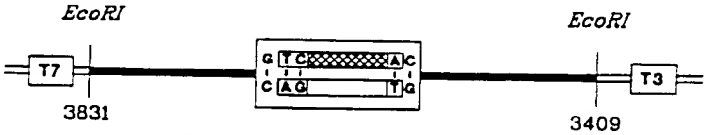
pPV12

T7 : (-) directed
PPV antizyme



pPV21

T3 : (-) directed
PPV antizyme



pPV22

T7 : (+) direected
PPV antizyme

Figure 5

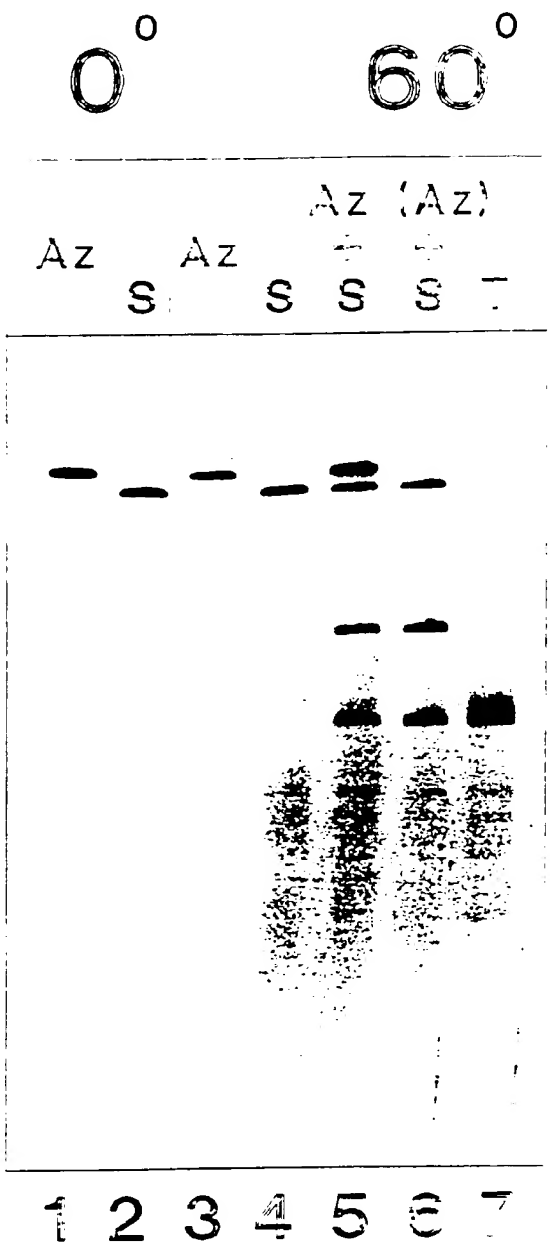


Figure 6

Ribozyme against the SalI recognition sequence modified to contain a StuI and XhoI sequence

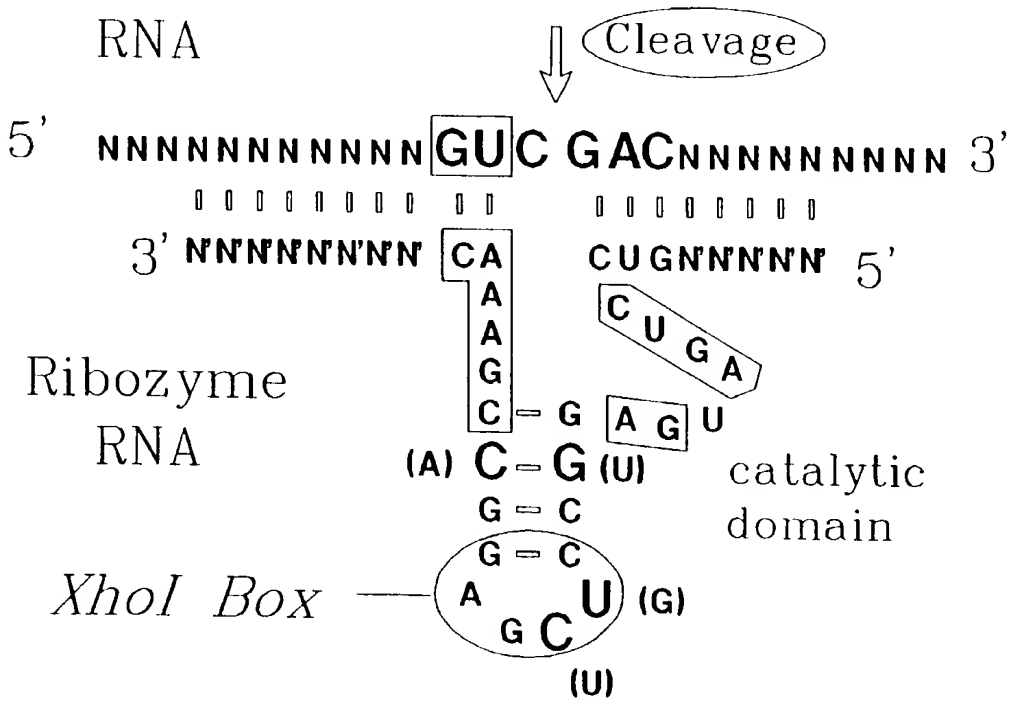


Figure 7

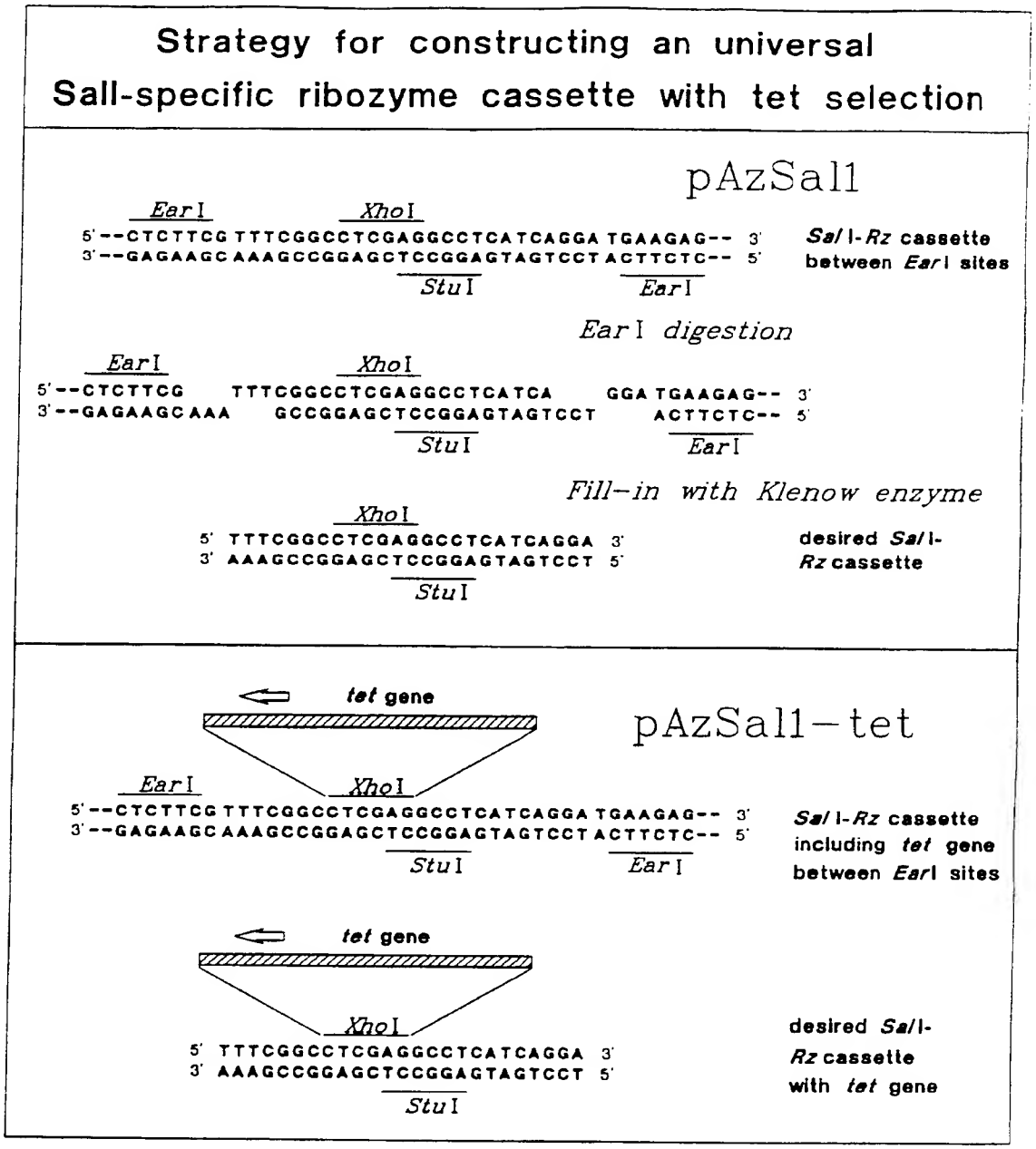

Strategy for constructing an universal
SalI-specific ribozyme cassette with tet selection
pAzSal1
EarI
XhoI
5'--CTCTTCG TTTCGGCCTCGAGGCCTCATCAGGA TGAAGAG-- 3'
3'--GAGAAGC AAAGCCGGAGCTCCGGAGTAGTCCT ACTTCTC-- 5'
StuI
EarI
SalI-Rz cassette between EarI sites
EarI digestion
EarI
XhoI
5'--CTCTTCG TTTCGGCCTCGAGGCCTCATCA GGA TGAAGAG-- 3'
3'--GAGAAGC AAA GCCGGAGCTCCGGAGTAGTCCT ACTTCTC-- 5'
StuI
EarI
Fill-in with Klenow enzyme
XhoI
5' TTTCGGCCTCGAGGCCTCATCAGGA 3'
3' AAAGCCGGAGCTCCGGAGTAGTCCT 5'
StuI
desired SalI-Rz cassette
tet gene
pAzSal1-tet
EarI
XhoI
5'--CTCTTCG TTTCGGCCTCGAGGCCTCATCAGGA TGAAGAG-- 3'
3'--GAGAAGC AAAGCCGGAGCTCCGGAGTAGTCCT ACTTCTC-- 5'
StuI
EarI
SalI-Rz cassette including tet gene between EarI sites
tet gene
XhoI
5' TTTCGGCCTCGAGGCCTCATCAGGA 3'
3' AAAGCCGGAGCTCCGGAGTAGTCCT 5'
StuI
desired SalI-Rz cassette with tet gene

Figure 8

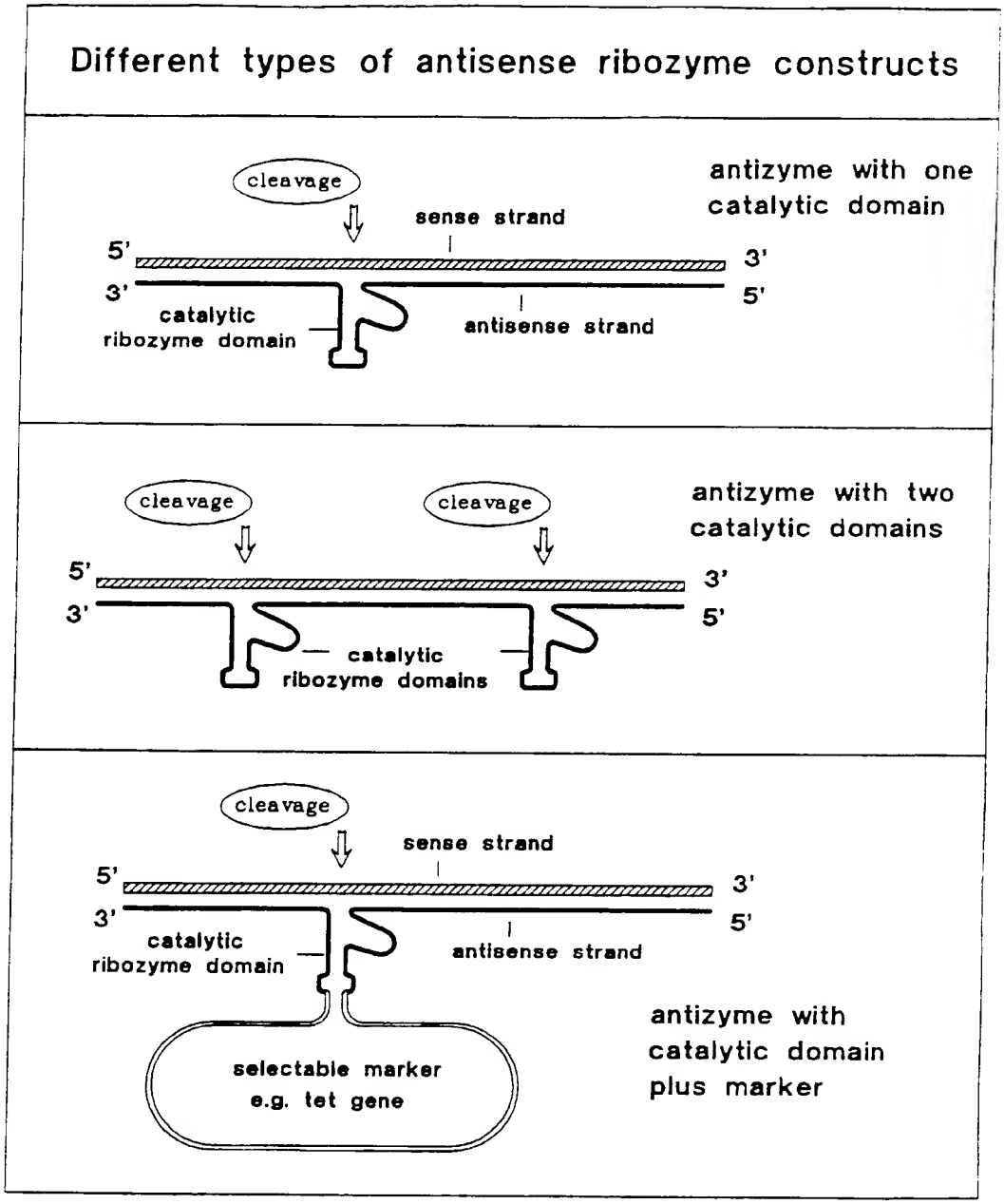

Different types of antisense ribozyme constructs
cleavage
sense strand
5'
3'
3'
5'
catalytic
ribozyme domain
antisense strand
antizyme with one
catalytic domain
cleavage
cleavage
5'
3'
3'
5'
catalytic
ribozyme domains
antizyme with two
catalytic domains
cleavage
sense strand
5'
3'
3'
5'
catalytic
ribozyme domain
antisense strand
selectable marker
e.g. tet gene
antizyme with
catalytic domain
plus marker

Figure 9

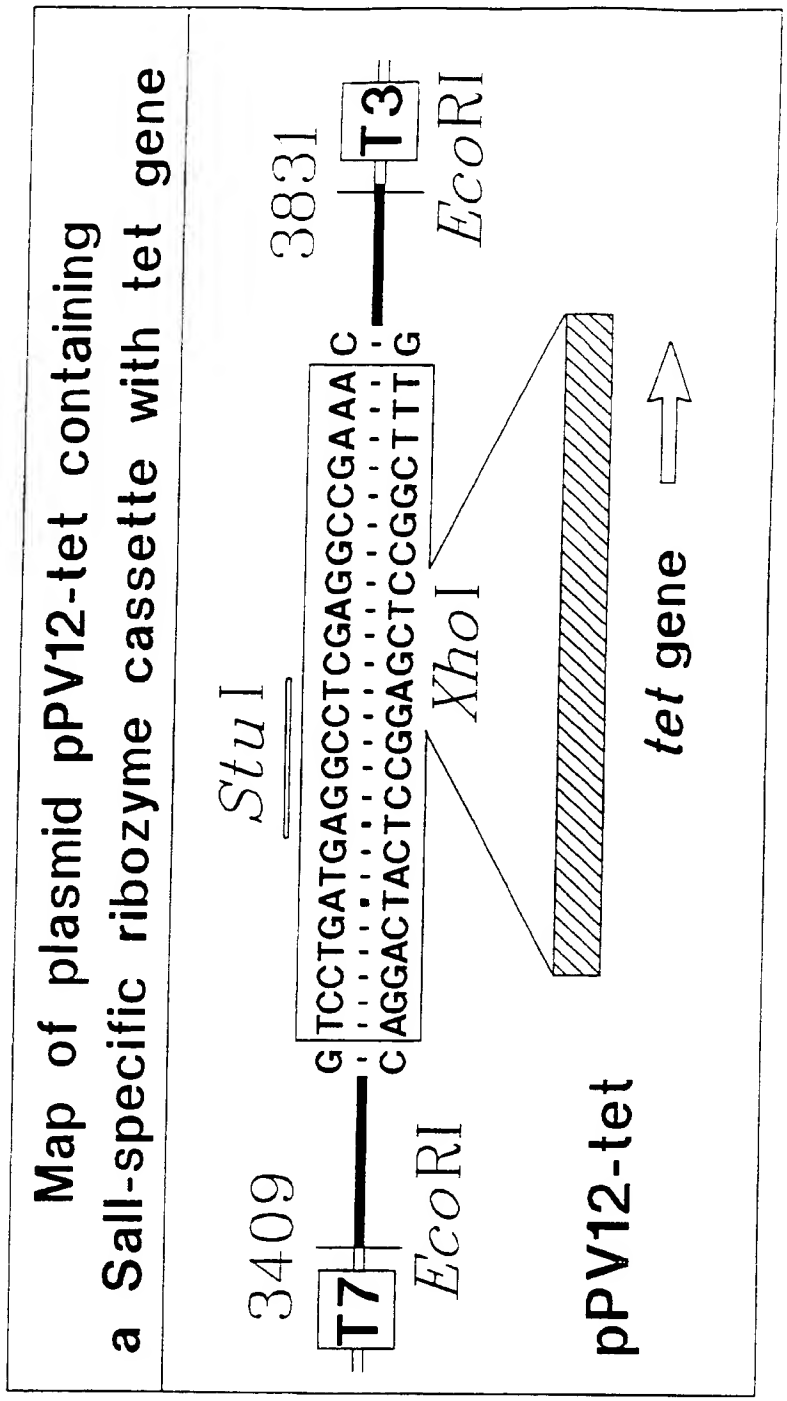

Map of plasmid pPV12-tet containing
a SalI-specific ribozyme cassette with tet gene
3409
T7
EcoRI
G
C
StuI
TCCTGATGAGGCCTCGAGGCCGAAAC
AGGACTACTCCGGAGCTCCGGCTTTG
XhoI
3831
T3
EcoRI
pPV12-tet
tet gene

Figure 10

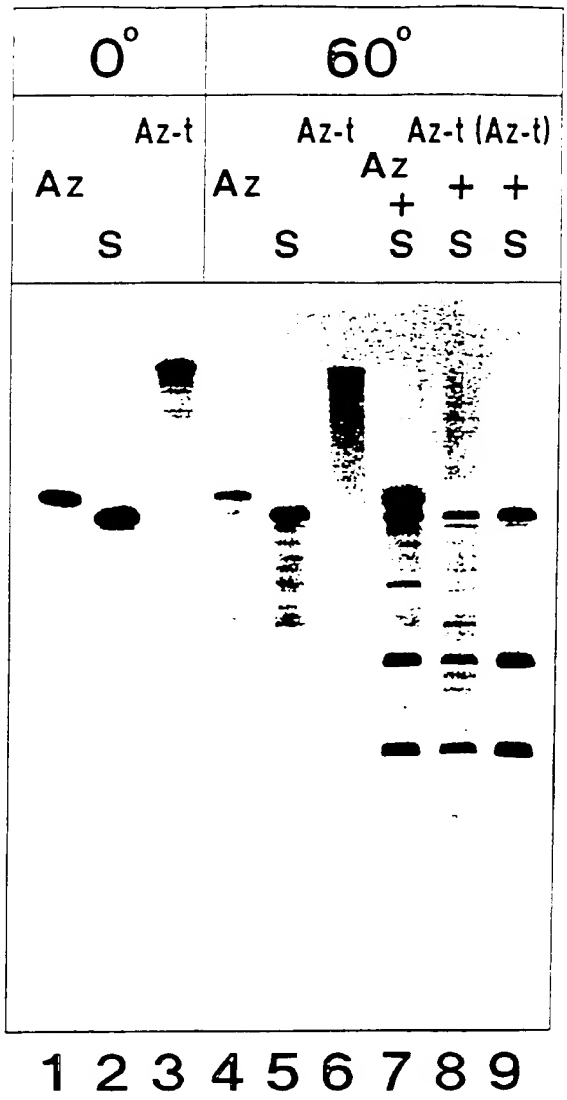


Figure 11

Generation of an excisable SalI-specific ribozyme cassette including the tet gene inserted into an AflII site

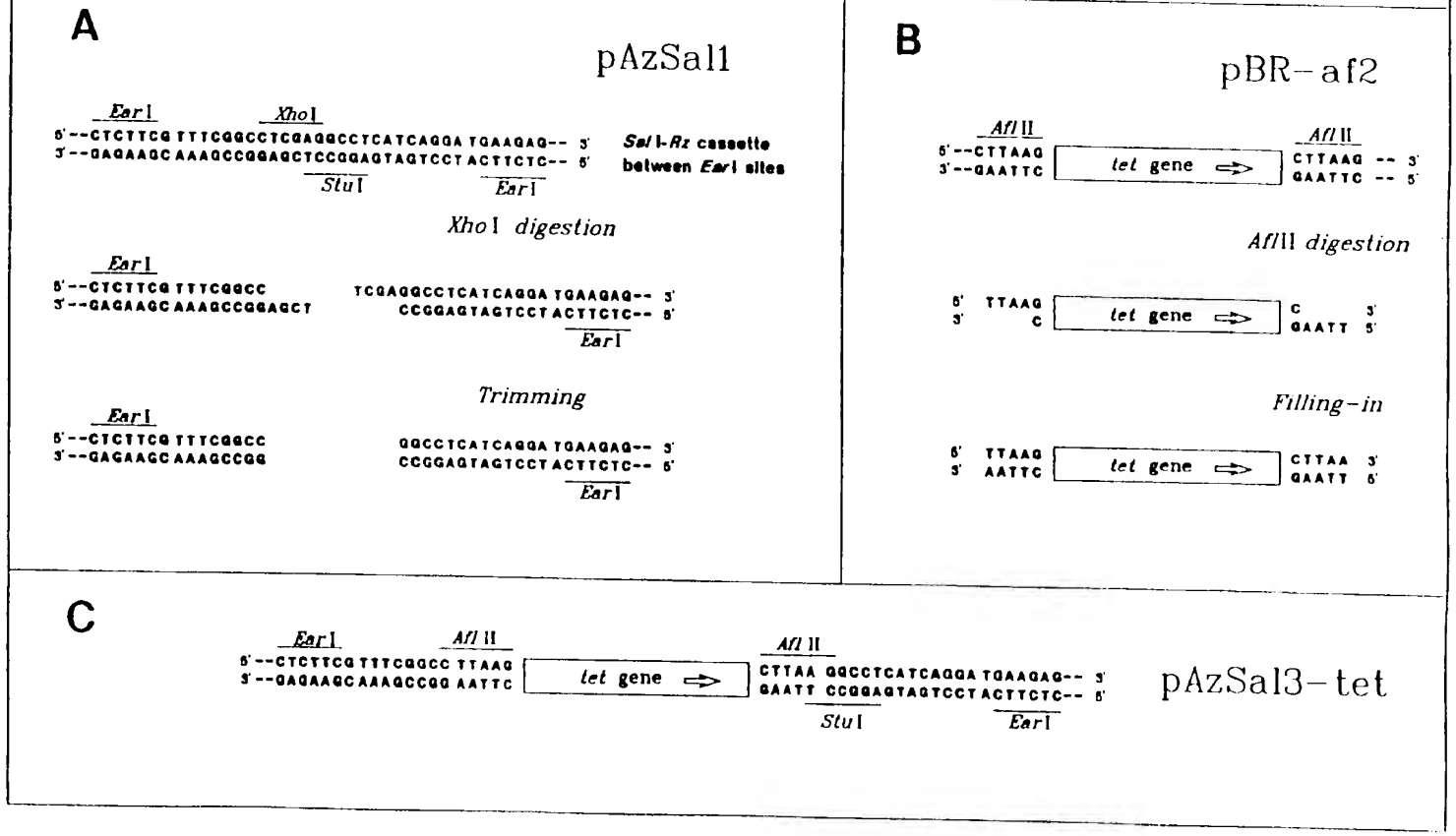


Figure 12

Ribozyme against the SalI recognition sequence modified to contain an AflII and XhoI sequence

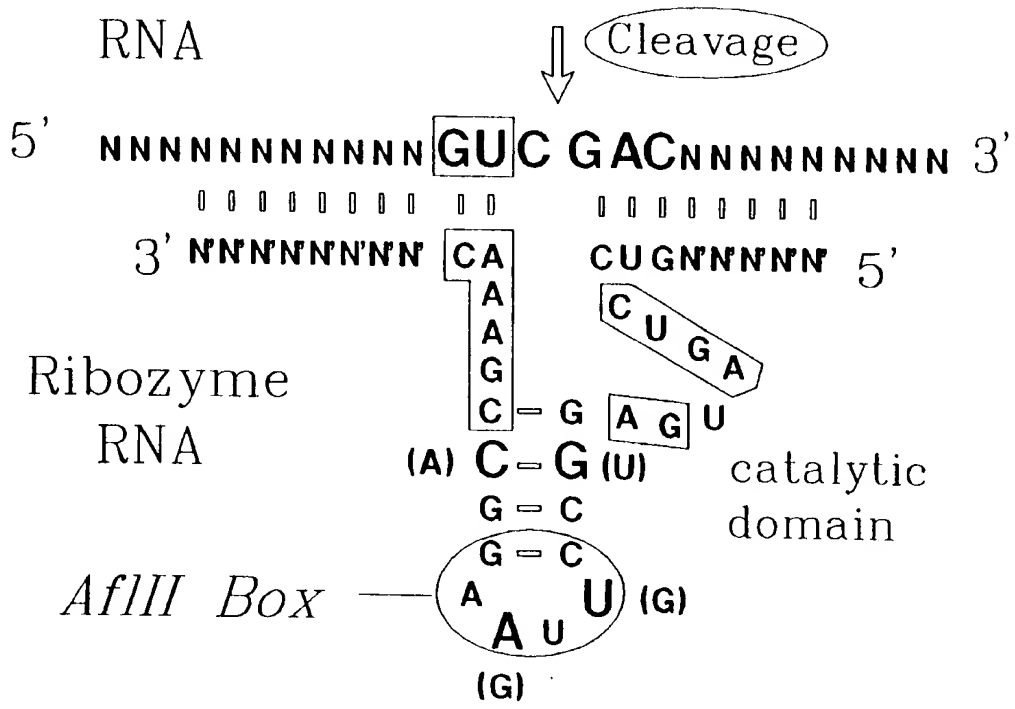


Figure 13

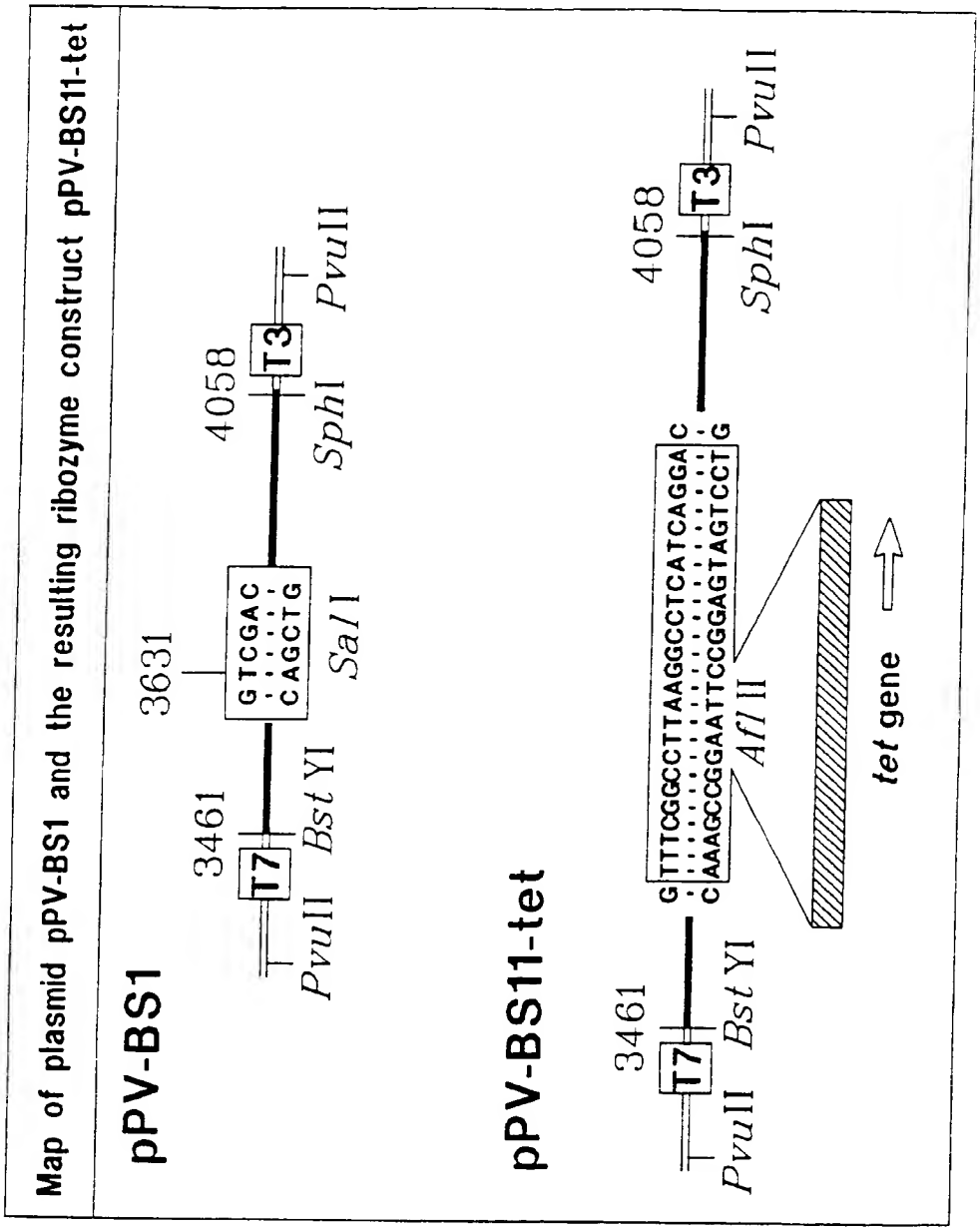

Map of plasmid pPV-BS1 and the resulting ribozyme construct pPV-BS11-tet
pPV-BS1
3631
3461
4058
T7
T3
GTCGAC
CAGCTG
PvuII
BstYI
SalI
SphI
PvuII
pPV-BS11-tet
3461
4058
T7
T3
GTTTCGGCCTTAAGGCCTCATCAGGAC
CAAAGCCGGAATTCCGGAGTAGTCCTG
PvuII
BstYI
AflII
SphI
PvuII
tet gene

Figure 14

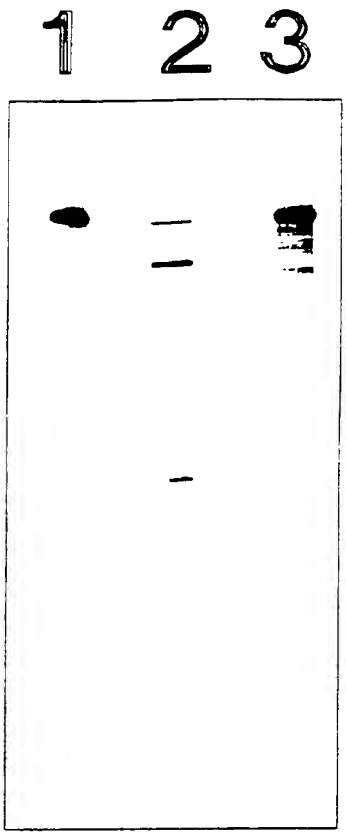


Figure 15

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/01403

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)*

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/11 C 12 N 9/00 C 12 N 15/10
C 12 N 1/21 A 61 K 48/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
P,X	Protein Engineering, vol. 3, no. 8, 1990, Oxford University Press, (GB), K. Taira et al.:" Construction of a novel artificial-ribozyme-releasing plasmid", pages 733-737, see the whole article, especially figure 2 ---	1-4,9-10,12-19
P,X	EP,A,0428881 (HOECHST AG) 29 May 1991, see page 2, lines 34-53; page 4, lines 40-55 ---	5-6
A	WO,A,8905852 (C.I.S.R.O.) 29 June 1989, see page 4, line 2 - page 11, line 13, & EP, A, 321201, (cited in the application) --- -/-	

* Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04-10-1991	24. 10. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Danielle van der Haas

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Journal of Cellular Biochemistry; Supplement 15D, 2 February-1 March 1991, abstract no. CD215, M. Tabler et al.:" Catalytic antisense RNAS by incorporation of specific ribozyme cassettes into cDNA", page 21, see the whole abstract	1-25

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons

1. [X] Claim numbers 26 because they relate to subject matter not required to be searched by this Authority, namely

Please see rule 39.1(iv) - PCT:
Methods for treatment of the human or animal body by surgery or therapy,as well as diagnostic methods.

2. [ ] Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers.

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. EP 9101403
SA 49684

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 22/10/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0428881	29-05-91	DE-A- 3935473	02-05-91
		AU-A- 6499590	02-05-91
WO-A- 8905852	29-06-89	AU-A- 2800789	19-07-89
		EP-A- 0321201	21-06-89